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TO THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Invention:	**Animal Food and Method**
Applicant:	Wilson, et al.
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on August 26, 2004

Rebecca Ball
(Signature)

Rebecca L. Ball
(Printed Name)

APPEAL BRIEF

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This is an appeal to the Board of Patent Appeals and Interferences from the Primary Examiner's February 26, 2004 final rejection of claims 1-6, 8-9, 13-20, 23, 25, 41, and 71-102. This appeal brief is submitted in triplicate along with a check for the $330.00 filing fee under 37 C.F.R. §1.17(c) in furtherance of the notice of appeal filed on May 26, 2004. Appellants hereby petition for a one-month extension of time under 37 C.F.R. §1.136(a) to file this response, extending the due date for response to August 26, 2004. Our check for $110.00 in payment of the extension fee under 37 C.F.R. § 1.17(a) is also enclosed. The Commissioner is hereby authorized to charge any additional fees or credit any

overpayment to Appellants' undersigned counsel's deposit account 10-0435 with reference to our matter 834460-68474. A duplicate copy of this authorization is enclosed for this purpose.

I. REAL PARTY IN INTEREST

The real party in interest is United Feeds, a corporation of the state of Indiana and having a business address of 4310 State Road 38 West, Sheridan, Indiana 46069. United Feeds is the owner, by assignment, of the entire interest in the subject application pursuant to an assignment recorded in the records of the Patent and Trademark Office at reel 012258, frame 0756.

II. RELATED APPEALS AND INTERFERENCES

A notice of appeal has been filed on July 21, 2004 in U.S. Application Serial No. 10/142,685, a divisional application of the present application.

III. STATUS OF THE CLAIMS

The subject application was originally filed with claims 1-70. In response to the April 23, 2002 office action, claims 21-22, 24, 26-40, 42-59, 63-64, 66, and 68 were canceled without prejudice and claims 71 and 72 were added. In response to the January 29, 2003 office action, claims 7, 60-62, 65, 67, and 69-70 were canceled without prejudice. In response to the June 2, 2003 office action, claims 10-12 were canceled without prejudice, and claims 73-102 were added. Thus, claims 1-6, 8-9, 13-20, 23, 25, 41, and 71-102 are pending. Claims 2, 9, 18, and 71-72 have been amended once. Claims 1, 19-20, 23, 25, and 41 have been amended three times. Claims 1-6, 8-9, 13-20, 23, 25, 41, and 71-102 are subject to a Final Rejection, from which this appeal was taken. An Appendix containing a list of the claims on appeal is attached to this appeal brief.

IV. STATUS OF AMENDMENTS

To Appellants' knowledge, there are no amendments that remain unentered.

V. SUMMARY OF THE INVENTION

The present invention relates to a method of increasing the reproductive performance of a female swine. The method includes the step of administering to the female swine a feed composition comprising a marine animal product wherein the marine animal product comprises C_{20} and C_{22} omega-3 fatty acids or esters thereof, and wherein the feed composition as a final mixture comprises about 0.025% to about 2% by weight of the marine animal product.

In illustrative embodiments, the marine animal product can be selected from a fish oil and a fish oil derived from a fish meal product, or a mixture thereof. In other illustrative embodiments the marine animal product can comprise a fish oil from a North Atlantic cold water fish, such as salmon oil. In another illustrative embodiment, the omega fatty acids can be stabilized by prilling. In yet another illustrative embodiment the feed composition can further comprise omega-6 fatty acids or esters thereof. In another exemplary embodiment, the omega-6 fatty acids/esters to omega-3 fatty acids/esters ratio in the feed composition as a final mixture can be from about 3:1 to about 20:1 (see page 12, lines 10-11). In another illustrative embodiment, the feed composition can further comprise an antioxidant. In another illustrative embodiment, the feed composition can further comprise a plant oil. In still other illustrative embodiments the feed composition as a final mixture can comprise about 0.025% to about 1% by weight of salmon oil or another fish oil or a fish oil derived from a fish meal product.

In another illustrative embodiment, the marine animal product is menhaden oil. In another embodiment, the feed composition as a final mixture comprises about 0.025% to about 1% by weight of menhaden oil.

In various illustrative embodiments, the feed composition can be administered to the female swine daily, daily for the lifetime of the female swine, beginning about 30 days before a first mating of the female swine during an estrus and continuing through a second mating of the female swine during the same estrus, beginning about 1 to about 4 days prior to parturition and continuing through the next breeding, or during lactation.

The present invention also relates to methods of increasing the number of live births to a female swine (see, for example, Figs. 1 and 2), of increasing the total number of births to a female swine, of increasing the uniformity of birth weight of offspring of a female swine, of increasing the farrowing rate of a female swine, and of increasing the reproductive performance of a breeding population of swine. Each of these methods includes the step of administering to the female swine a feed composition comprising a marine animal product wherein the marine animal product comprises C_{20} and C_{22} omega-3 fatty acids or esters thereof, and wherein the feed composition as a final mixture comprises about 0.025% to about 2% by weight of the marine animal product.

The present invention also relates to a method of increasing the reproductive performance of a female swine, comprising the step of administering to the female swine a feed composition comprising a marine animal product wherein the marine animal product comprises C_{20} omega-3 fatty acids or esters thereof, and wherein the feed composition as a final mixture comprises about 0.025% to about 2% by weight of the marine animal product.

The invention further relates to a method of increasing the reproductive performance of a female swine, comprising the step of administering to the female swine a feed composition comprising a marine animal product wherein the marine animal product

comprises C_{22} omega-3 fatty acids or esters thereof, and wherein the feed composition as a final mixture comprises about 0.025% to about 2% by weight of the marine animal product.

The present invention also relates to a method of increasing the reproductive performance of a female swine, comprising the step of administering to the female swine a feed composition comprising a marine animal product wherein the marine animal product is a fish meal product and wherein the fish meal product comprises C_{20} and C_{22} omega-3 fatty acids or esters thereof. In one illustrative embodiment, the fish meal product is from a North Atlantic cold water fish. In another illustrative embodiment, the feed composition further comprises omega-6 fatty acids or esters thereof. In another embodiment, the omega-6 fatty acids/esters to omega-3 fatty acids/esters ratio in the feed composition as a final mixture is from about 3:1 to about 20:1. In yet another illustrative embodiment, the feed composition as a final mixture comprises about 1% to about 10% by weight of the fish meal product. In another illustrative embodiment, the feed composition as a final mixture further comprises an antioxidant. In still other embodiments, the feed composition is administered daily to the female swine, beginning about 30 days before a first mating of the female swine during an estrus and continuing through a second mating of the female swine during the same estrus, beginning about 1 to about 4 days prior to parturition and continuing through the next breeding, or during lactation (see page 9, line 21 through page 10, line 7).

The present invention also relates to a method of increasing the reproductive performance of a female swine, comprising the step of administering to the female swine a feed composition comprising a marine animal product wherein the marine animal product comprises omega-6 fatty acids or esters thereof and C_{20} and C_{22} omega-3 fatty acids or esters thereof, and wherein the omega-6 fatty acids/esters to omega-3 fatty acids/esters ratio in the feed composition as a final mixture is from about 3:1 to about 20:1. In one illustrative embodiment, the marine animal product is an oil from a North Atlantic cold water fish, such

as salmon oil. In other embodiments, the feed composition as a final mixture comprises about 0.025% to about 1% or about 0.025% to about 2% by weight of salmon oil. In yet other illustrative embodiments, the feed composition as a final mixture comprises about 0.025% to about 1% or about 0.025% to about 2% by weight of the marine animal product. In another embodiment, the feed composition as a final mixture further comprises an antioxidant. In yet another embodiment, the omega fatty acids in the marine animal product are stabilized by prilling. In another illustrative embodiment, the marine animal product is menhaden oil, and in related illustrative embodiments, the feed composition as a final mixture comprises about 0.025% to about 1% or about 0.025% to about 2% by weight of menhaden oil.

In various illustrative embodiments, the feed composition is administered daily to the female swine, beginning about 30 days before a first mating of the female swine during an estrus and continuing through a second mating of the female swine during the same estrus, beginning about 1 to about 4 days prior to parturition and continuing through the next breeding, or during lactation.

VI. ISSUES PRESENTED ON APPEAL

The first issue is whether claims 1-6, 8-9, 13-20, 23, 25, 41, and 71-102 are unpatentable under 35 U.S.C. § 103(a) over Fritsche et al. in view of Boudreaux et al. The second issue is whether claims 1-6, 8-9, 13-20, 23, 25, 41, and 71-102 are unpatentable under 35 U.S.C. § 103(a) over Abayasekara et al.

VII. GROUPING OF THE CLAIMS

Claims 1-6, 8-9, 13-20, 23, 25, 41, and 71-102 are all separately patentable at least for the reasons stated in the following **ARGUMENTS**.

VIII. ARGUMENTS

A. Rejection of claims 1-6, 8-9, 13-20, 23, 25, 41, and 71-102 under 35 U.S.C. § 103(a) over Fritsche et al. in view of Boudreaux et al.

Claims 1-6, 8-9, 13-20, 23, 25, 41, and 71-102 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Fritsche et al. in view of Boudreaux et al. A copy of each cited reference is attached hereto for the Board's convenience. It is Appellants' position that neither Fritsche et al. nor Boudreaux et al., alone or in combination, teach or suggest the subject matter of claims 1-6, 8-9, 13-20, 23, 25, 41, and 71-102, and that these claims are, therefore, patentably distinct from the prior art of record.

Rejection of claim 1

With regards to Appellants' claim 1, the Examiner cites Fritsche et al. as disclosing that menhaden fish oil is useful in dietary compositions to feed to sows "including gestation until farrowing and lactation and methods of the treatment to benefit sow's performance in maternal period including gestation until farrowing and lactation, and benefit pig survival, number of pigs born per sow, birth weight and weaning weights (see title of the article, abstract, Introduction, and working examples in "Animals and Diets" at the right column of page 1841 to the left column of page 1842)." See page 3, lines 12-17 of the February 26, 2004 office action. The Examiner indicates that "one having ordinary skill in the art at the time the invention was made would have been motivated to optimize the amount of fish oils to 0.025% to 2% by weight in the prior art compositions" of Fritsche et al. (page 4, lines 15-17 of the February 26 office action). The Examiner cites Boudreaux et al. as disclosing "the ratio of omega-6 fatty acids to omega-3 fatty acids" that is within the claims of the present application (page 4, lines 8-9 of the February 26 office action).

Claim 1 is directed to "a method of increasing the reproductive performance of a female swine" by administering to the female swine a feed composition comprising a marine animal product wherein the marine animal product comprises C_{20} and C_{22} omega-3 fatty acids or esters thereof and wherein the feed composition as a final mixture comprises about 0.025% to about 2% by weight of the marine animal product. Fritsche et al. teaches supplementing the diet of pregnant sows with 3.5% or 7% by weight of menhaden fish oil to simply enrich the amount of omega-3 fatty acids in the suckling pigs, obtained through the sow's milk. As stated in the last sentence of the Introduction, "[t]he primary objective of this study was to determine whether neonatal pigs could be supplied with *n*-3 PUFA by feeding a source of these fatty acids to the sow." Fritsche et al. teaches nothing more.

As disclosed in Fritsche et al., the three experimental diets fed to sows on day 107 of gestation were diets in which menhaden fish oil was used as a substitute for lard at 0, 3.5%, and 7% of the diet (see page 1841 of Fritsche et al., first sentence of the "Materials and Methods"). The diets are designated as LA (no menhaden fish oil and 7% lard), MIX (3.5% lard and 3.5% menhaden fish oil), and FO (7% menhaden fish oil). See page 1841 of Fritsche et al., first sentence of the "Materials and Methods." As stated above, the objective of this study was to determine whether neonatal pigs could be supplied with omega-3 fatty acids by feeding a source of these fatty acids to the sow. The study demonstrates that when a source of omega-3 fatty acids (*i.e.*, menhaden fish oil) is fed to the sow, the level of omega-3 fatty acids is increased in the sow's serum and in the sow's milk and that the level of omega-3 fatty acids is also increased in the serum of the suckling pigs, presumably largely through the sow's milk. As stated in the last sentence of the Abstract, "[i]n conclusion, we have demonstrated that feeding FO to sows during late gestation and lactation enriches the newborn pig with *n*-3 PUFA." See also the sections of the "Results and Discussion" titled "Serum Fatty Acid Profiles of Sows," "Milk Fatty Acid Profiles," and "Serum Fatty Acid

Profiles of Pigs." Accordingly, this reference teaches only that when a source of omega-3 fatty acids (*i.e.*, menhaden fish oil) is fed to the sow, the level of omega-3 fatty acids is increased in the sow's serum and milk and that, as a result, the level of omega-3 fatty acids is increased in the serum of the suckling pigs. Fritsche et al. does not teach, suggest, or even mention that reproductive performance is increased in sows fed a diet supplemented with omega-3 fatty acids.

In fact, Fritsche et al. teaches away from the invention of claim 1. In the "Results and Discussion" section of Fritsche et al. in the section titled "Litter Size, Birth Weights, and Weaning Weights," the effects of feeding the LA, MIX, and FO diets to sows on litter size, birth weights of pigs, and weaning weights of pigs is described. In the first paragraph of this section (page 1843, column 1, paragraph 2), Fritsche et al. reads as follows:

> There were no significant differences in feed consumption or weight loss by sows among treatment groups (data not shown). Similarly, the number of live pigs born per litter (9.4, 11.8, and 8.2 ± 1.2; $P = .29$; n = 5, 4, and 6, for LA, MIX, and FO groups, respectively) and pig birth weights (1.51, 1.42, and 1.50 ± .05 kg; $P = .25$, for LA, MIX, and FO groups, respectively) **did not** differ among treatment groups. Weaning weights for the MIX group were lower ($P < .007$) than those for the other groups (13.5 vs 16.8 and 16.0 ± .7 kg for MIX, LA, and FO, respectively). However, differences in age at weaning (28.8, 25.6, and 26.8 ± .33 d) accounted for most of this disparity in weaning weights between diet groups. When these data were analyzed with age of weaning as a covariate, **no diet effect was noted** ($P < .05$).

(Emphasis added). Accordingly, Fritsche et al. teaches that administering to pregnant sows a feed composition supplemented with 3.5% or 7% by weight of menhaden fish oil **does not** increase the number of live pigs born per litter, **does not** increase birth weights, and **does not** increase weaning weights. Clearly, Fritsche et al. teaches away from "a method of increasing the reproductive performance of a female swine" as claimed in Appellants' claim 1 and cannot render obvious the subject matter of claim 1.

The Examiner has rejected all of the pending claims over Fritsche et al. in view of Boudreaux et al. Boudreaux et al. is relied on by the Examiner to provide the teaching of a range of ratios of omega-3 fatty acids to omega-6 fatty acids. Even though all of the pending claims do not specify a ratio of omega-3 fatty acids to omega-6 fatty acids, such as the ratio specified in, for example, claim 6, the Examiner has rejected all of the pending claims over Fritsche et al. in view of Boudreaux et al.

The Examiner's rejection of claim 1, and any other claim that **does not** specify a ratio of omega-3 fatty acids to omega-6 fatty acids (claims 2-5, 8-9, 13-20, 23, 25, 41, 71-75, 77-82, 96-97, and 101-102), under 35 U.S.C. § 103(a) over Fritsche et al. in view of Boudreaux et al. is improper. For claims that do specify a ratio, Boudreaux et al. does nothing to overcome the insufficiencies of Fritsche et al. because Boudreaux et al. does not teach or suggest "a method of increasing the reproductive performance of a female swine." Rather, Boudreaux et al. describes the results of tests to determine the effect of varying the ratio of omega-3 to omega-6 fatty acids in dog food on inflammation and thrombosis in dogs. Thus, Boudreaux et al. does not overcome the insufficiencies of Fritsche et al. because Boudreaux et al. does not provide the teaching of "a method of increasing the reproductive performance of a female swine" that is lacking in Fritsche et al.

Claim 1 specifies that the feed composition used in the claimed method comprises about 0.025% to about 2% by weight of the marine animal product. The Examiner's arguments, based on the knowledge of the skilled artisan, with respect to optimization of the amount of the marine animal product specified in claim 1 fail because neither Fritsche et al. nor Boudreaux et al. suggest optimizing the **amount of oil** used in the compositions described in those references. Furthermore, neither reference suggests optimizing to obtain a weight percentage of the oil from "about 0.025% to about 2%" of the final feed composition. Thus, the Examiner's arguments with respect to optimization are

misplaced. Accordingly, based on all of the above arguments, the Examiner has failed to establish, for claim 1, a case of prima facie obviousness under 35 U.S.C. § 103(a) over Fritsche et al. in view of Boudreaux et al.

Moreover, there is no motivation in Fritsche et al. or Boudreaux et al. to combine these references. There is no motivation for a person of ordinary skill in the art to combine Fritsche et al., disclosing a feed composition study for pregnant sows where the study noted that there was no effect on litter size, birth weights, or weaning weights (*i.e.*, no reproductive effects), with a reference describing a dog food composition study which concludes that "[t]he diet used in this study did not have a clinically significant effect on platelet reactivity, coagulation protein screening assays, fibrinogen, or antithrombin II activity in purpose-bred laboratory beagles" (see page 241 of Boudreaux et al) to reach Appellants' claimed invention directed to "a method of increasing reproductive performance." Neither Fritsche et al. or Boudreaux et al. teach or suggest any successful reproductive performance study of a female swine. Fritsche et al. and Boudreaux et al. do not combine to teach or suggest the subject matter of Appellants' claims. Furthermore, there is no mention or suggestion in Fritsche et al. of optimizing omega-3 to omega-6 fatty acid ratios to achieve the results sought in Fritsche et al., and there is no mention or suggestion in Boudreaux et al. of optimizing omega-3 to omega-6 fatty acid ratios for use in pregnant animals for any reason, in particular for use in pregnant sows. Thus, a skilled artisan would not be motivated to look to Boudreaux et al. to modify Fritsche et al.

An Examiner cannot simply cite different features that are claimed without explaining the motivation to combine or modify the references. *In re Fritch*, 972 F.2d 1260, 23 USPQ2d 1780 (Fed. Cir. 1992). It is the Examiner's burden to explain the motivation to combine the references and she has not done so. The Examiner has stated that "[o]ne having ordinary skill in the art at the time the invention was made would have been motivated to

optimize the particular ratio of omega-6 fatty acids to omega-3 fatty acids herein in the compositions herein, since the range of the ratio of omega-6 fatty acids to omega-3 fatty acids herein in the composition to be administered to animals is known according to Boudreaux et al." (see page 4, line 19 through page 5, line 2 of the February 26 office action). The above-quoted statement does not explain the motivation to combine Fritsche et al. with Boudreaux et al. For all of the above reasons, the Examiner's prima facie case fails with respect to the rejection of claim 1 under 35 U.S.C. § 103(a) over Fritsche et al. in view of Boudreaux et al.

Rejection of independent claims 19, 20, 23, 25, 41, 71-73, and 83

Claims 19, 20, 23, 25, 41, 71-73, and 83 are the remaining independent claims in the application. Independent claims 41, 71, 72, and 83 differ from claim 1 only in that these claims specify "a method of increasing the reproductive performance of a breeding population of swine," that the marine animal product comprises "C_{20} omega-3 fatty acids," that the marine animal product comprises "C_{22} omega-3 fatty acids," and that the marine animal product comprises "omega-6 fatty acids or esters thereof and C_{20} and C_{22} omega-3 fatty acids," respectively. Appellants submit that claims 41, 71, 72, and 83 are patentable for the same reasons as noted above with respect to claim 1. Also, Appellants wish to point out that Fritsche et al. and Boudreaux et al. do not teach or suggest the limitation of "a method of increasing the reproductive performance of a breeding population of swine," as recited in claim 41.

Independent claims 19 and 20 are directed to "a method of increasing the number of live births" and "a method of increasing the total number of births," to female swine, respectively. The arguments discussed above with respect to claim 1 apply to the rejection of claims 19 and 20 under 35 U.S.C. § 103(a) over Fritsche et al. in view of Boudreaux et al. Furthermore, Fritsche et al., which teaches that administering to pregnant

sows a feed composition supplemented with menhaden fish oil **does not** increase the number of live pigs born per litter, clearly does not render obvious "a method of increasing the number of live births" or "a method of increasing the total number of births" as claimed in claims 19 and 20, respectively.

Independent claims 23, 25, and 73 differ from claim 1 because these claims specify "a method of increasing uniformity of birth weight," "a method of increasing the farrowing rate," and that the marine animal product is "a fish meal product," respectively. The arguments discussed above for claim 1 apply with equal force to the rejection of claims 23, 25, and 73 under 35 U.S.C. § 103(a) over Fritsche et al. in view of Boudreaux et al. Moreover, Fritsche et al. and Boudreaux et al., either alone or in combination, do not teach, disclose, or suggest 1.) **increasing** the uniformity of birth weight and, thus, do not teach "a method of increasing uniformity of birth weight," 2.) **increasing the farrowing rate**, and, thus, do not teach "a method of increasing the farrowing rate," or 3.) **a fish meal** product, and, thus, do not teach that the marine animal product is "a fish meal product." Therefore, it is Appellants' position that the Examiner has also failed to establish a case of prima facie obviousness for independent claims 19, 20, 23, 25, 41, 71-73, and 83 under 35 U.S.C. § 103(a) over Fritsche et al. in view of Boudreaux et al.

Rejection of dependent claims 2-6, 8-9, 13-18, 74-82, and 84-102

Claims 2-6, 8-9, 13-18, 96-97, and 101-102 depend, directly or indirectly, from claim 1. Claims 74-82 depend, directly or indirectly, from claim 73. Claims 84-95 and 98-100 depend, directly or indirectly, from claim 83. All of the arguments discussed above for the independent claims from which these claims depend apply with equal force to the rejection of the corresponding dependent claims. Moreover, Fritsche et al. and Boudreaux et al., either alone or in combination, do not teach, disclose, or suggest 1.) the limitations in

dependent claims 4, 8, 85, 86, and 88 specifying that the marine animal product comprises salmon oil, 2.) the limitations in dependent claims 14-15, 79-80, 91-92, and 102 specifying regimens for administration of the feed composition to the female swine, or 3.) the limitations in dependent claims 18 and 95 specifying that the fatty acids are stabilized by prilling. Accordingly, the Examiner has also failed to establish a case of prima facie obviousness for dependent claims 2-6, 8-9, 13-18, 74-82, and 84-102 under 35 U.S.C. § 103(a) over Fritsche et al. in view of Boudreaux et al. Appellants further point out that claims 14, 79, and 91 recite the step that the "feed composition is administered to the female swine beginning about 30 days before a first mating of the female swine during an estrus and continuing through a second mating of the female swine during the same estrus," which step is not taught or suggested in Fritsche et al., Boudreaux et al. or any combination thereof. Also, claim 102 recites the step that the "feed composition is fed to the female swine daily for the lifetime of the female swine," which step is not taught or suggested in Fritsche et al., Boudreaux et al., or any combination thereof. In addition claims 15, 80, and 92 recite that the "feed composition is administered to the female swine beginning about 1 to about 4 days prior to parturition and continuing through the next breeding," which step is not taught or suggested in Fritsche et al., Boudreaux et al., or any combination thereof. Fritsche et al. simply teaches feeding the composition described in Fritsche et al. beginning at day 107 of gestation (about 2 weeks before the female gives birth) through the subsequent lactation period.

B. <u>Examiner's arguments regarding Appellants' alleged admissions and unsupported statements of the Examiner</u>.

The final office action misrepresents Appellants' position on several matters. On page 5, line 11 through page 6, line 7 of the February 26, 2004 office action, it is incorrectly stated that:

> Additionally, omega-3 fatty acids in particular are known to be useful to increase female animal fertility (see Applicant's admission regarding the prior art at page 2 lines 29-30 of the specification). Omega-6 fatty acids are known to increase the number of live births in animals (see page 2 lines 24-25 of the specification). It is noted that Applicant clearly cited the prior art references, i.e., journal articles and patents, for these prior art teachings in the specification. Further, salmon oil or menhaden oil is well known to contain C_{20} and C_{22} omega-3 fatty acids and omega-6 fatty acids are known to benefit female swine performance. Therefore, one of ordinary skill in the art would have found it obvious to employ salmon oil or menhaden oil as fish oil and determine the particular amounts (percentage) of fish oil and the time for the administration such as about 30 days before a first mating through a second mating based the prior art teachings. Furthermore, one of ordinary skill in the art would have found it obvious to stabilize the fish oil in the feed by prilling since prilling is known as an art recognized technique for stabilizing fish oil. Thus the claimed invention as a whole is clearly prima facie obvious over the combined teachings of the prior art.

The Examiner's argument is based on her incorrect assertion that Appellants have made two admissions. First, the Examiner incorrectly states that Appellants have admitted that "omega-3 fatty acids in particular are known to be useful to increase female animal fertility (see Applicant's admission regarding the prior art at page 2 lines 29-30 of the specification)." See page 5, lines 11-13 of the February 26 office action. Second, the Examiner states that Appellants have admitted that "omega-6 fatty acids are known to increase the number of live births in animals (see page 2 lines 24-25 of the specification)." See page 5, lines 13-14 of the February 26 office action. Page 2 of Appellants' specification cites prior art that Appellants are aware of. The final office action improperly asserts that Appellants have made admissions. The final office action is improper for misconstruing Appellants' description of the prior art rather than fairly evaluating the prior art and, if applicable, relying on what is taught in such prior art.

The Examiner states that the Appellants have admitted on page 2, lines 29-30 of Appellants' specification that "omega-3 fatty acids in particular are known to be useful to increase female animal fertility." The Examiner has misstated Appellants' specification. Page 2, lines 29-30 of Appellants' specification states that "the effect of linseed oil, and

omega-3 fatty acids in particular, on increased sperm fertility and female fertility, applicable to cattle, sheep, and rats **has been studied**." (Emphasis added). Clearly, this statement is not an admission that "omega-3 fatty acids in particular are known to be useful to increase female animal fertility." Accordingly, the Examiner badly misstated Appellants' specification.

The Examiner misquoted the same statement in Appellants' specification in the office actions mailed on April 23, 2002 (see page 5, lines 6-7) and January 29, 2003 (see page 4, lines 18-20). Appellants have indicated to the Examiner that the Examiner misquoted Appellants' statement in the responses filed on October 21, 2002 (page 14, paragraph 1) and May 8, 2003 (page 19, paragraphs 1 and 2) and in the interview conducted on May 5, 2003 (see page 9, paragraph 1 of the May 8, 2003 response). It is Appellants' position that it is improper for the Examiner to misquote Appellants' specification and to attempt to use a misstatement as an admission against the Appellants when the Appellants have made no such admission.

Furthermore, the statement in Appellants' specification, referred to by the Examiner, simply indicates that the effect of linseed oil, and omega-3 fatty acids on female fertility, applicable to cattle, sheep, and rats **has been studied**, and suggests nothing about the results of those studies. A statement that an effect on fertility **has been studied** in cattle, sheep, and rats, with no indication what that effect may be, cannot render obvious Appellants' claims to methods of increasing the reproductive performance of a female swine.

As a further basis for her argument (page 5, line 11 through page 6, line 7 of the February 26 office action), the Examiner also states that Appellants have admitted on page 2, lines 24-25 of Appellants' specification that that "omega-6 fatty acids are known to increase the number of live births in animals." Again the Examiner has misstated Appellants' specification. Page 2, lines 24-25 of Appellants' specification states that "linseed and corn oil have been used in animal feed as a source of omega-6 fatty acids to increase the number

of weaned rats." See page 2, lines 24-25 of Appellants' specification and the amendment made to the specification on page 2 of the October 21, 2002 response. The Examiner misquoted the same statement in Appellants' specification in the office actions mailed on April 23, 2002 (see page 5, lines 4-5) and January 29, 2003 (see page 4, lines 16-17). Appellants have indicated to the Examiner that the Examiner misquoted Appellants' statement in the responses filed on October 21, 2002 (page 17, first full paragraph) and May 8, 2003 (page 20, last paragraph). Again, Appellants believe that it is improper for the Examiner to misquote Appellants' and to attempt to use a misstatement as an alleged admission.

Furthermore, this statement indicates that **linseed and corn oil** have been used as a source of omega-6 fatty acids to increase the number of weaned rats. Each of the claims in the instant application requires that the feed composition used in the claimed method contains "C_{20} and C_{22} omega-3 fatty acids or esters thereof ." Linseed oil and corn oil are plant oils and, although linseed oil and corn oil may contain shorter chain fatty acids, it is well-known that plant oils do not contain C_{20} and C_{22} fatty acids or esters thereof, but animal oils do contain these longer chain fatty acids (see page 8 of the response filed on October 21, 2002 and pages 14-16 of the response filed on May 8, 2003 and the declarations, cited references, and exhibits referenced therein). Accordingly, the statement referenced, but misquoted by the Examiner, cannot render obvious Appellants' claims.

To further support her argument (page 5, line 11 through page 6, line 7 of the February 26 office action), the Examiner states that "C_{20} and C_{22} omega-3 fatty acids and omega-6 fatty acids are known to benefit female swine performance" (page 5, lines 18-19 of the February 26 office action). The Examiner cites no support for this statement. The only reference cited by the Examiner in the instant application that is directed to reproductive performance of female swine is Stitt et al. (reference AF). Stitt et al. describes the use of

flaxseed in a feed composition to increase the reproductive performance of female swine. As stated above, it is well-known that plant oils do not contain C_{20} and C_{22} fatty acids or esters thereof (see page 8 of the response filed on October 21, 2002 and pages 14-16 of the response filed on May 8, 2003 and the declarations, cited references, and exhibits referenced therein). Thus, there is no support on the record for the Examiner's statement that "C_{20} and C_{22} omega-3 fatty acids and omega-6 fatty acids are known to benefit female swine performance" and the Examiner cites no support for this statement. Accordingly, the Examiner's use of this statement is clearly improper.

The Examiner's arguments on page 5, line 11 through page 6, line 7 of the February 26, 2004 office action are based on misstatements of the Appellants' specification and unsupported statements of the Examiner. Moreover, the statements in Appellants' specification, referred to, but misquoted by the Examiner, do not render obvious Appellants' claims. Accordingly, the Examiner has not established a case of prima facie obviousness based on her arguments on page 5, line 11 through page 6, line 7 of the February 26, 2004 office action.

C. Rejection of claims 1-6, 8-9, 13-20, 23, 25, 41, and 71-102 under 35 U.S.C. § 103(a) over Abayasekara et al.

The Examiner has rejected claims 1-6, 8-9, 13-20, 23, 25, 41, and 71-102 under 35 U.S.C. § 103(a) over Abayasekara et al. A copy of the cited reference is attached hereto for the Board's convenience. It is Appellants' position that Abayasekara et al. does not teach or suggest the subject matter of claims 1-6, 8-9, 13-20, 23, 25, 41, and 71-102, and that these claims are, therefore, patentably distinct over Abayasekara et al.

Rejection of claim 1

With regards to Appellants' claim 1, the Examiner indic

ates (page 6, lines 13-17 of the February 26 office action) that Abayasekara et al. discloses "that dietary fatty acid compositions, i.e., fish oil, comprising instant fatty acids such as omega-6 fatty acids to omega-3, and the [sic] their ratio of (see particularly Fig 1 at page 277) are useful in increasing the female performance, i.e., follicular development in the ovary, ovaulation [sic], corpus luteum function, pregnancy, parturition, and lactation (see abstract, page 279-282)."

Appellants' claim 1 is directed to a method of increasing the reproductive performance of a "female swine" and requires that the marine animal product in the feed composition used in the claimed method comprises "C_{20} and C_{22} omega-3 fatty acids." No where does Abayasekara et al. teach or suggest adding "C_{20} and C_{22} omega-3 fatty acids" to the diets of female swine to increase the reproductive performance of "female swine." Appellants ask that the Examiner meet her burden and point to such a statement in Abayasekara et al. that teaches or suggests that "C_{20} and C_{22} omega-3 fatty acids" are effective to increase the reproductive performance of "female swine."

Abayasekara et al. describes studies to determine the effects of C_{18} omega-3 and omega-6 fatty acids, not of "C_{20} and C_{22} omega-3 fatty acids" on the reproductive performance of ruminants. Swine are not ruminants. Abayasekara et al. focuses on ruminants because the particular microorganisms present in the rumen of these animals cause extensive hydrogenation of unsaturated fatty acids (see page 276, last sentence of column 2 of Abayasekara et al.) and, consequently, even though the normal diet of ruminants contains predominantly unsaturated fatty acids, the fat content of the blood, tissues, and milk is highly saturated. Therefore, Abayasekara et al. suggests that the dietary supply of unsaturated fatty acids (*i.e.*, C_{18} omega-3 and omega-6 fatty acids) should be increased in ruminants.

Abayasekara et al. states (see page 276, column 2, first sentence of last paragraph) that "[t]he fatty acid composition of blood, tissue and milk in non-ruminants generally reflects the fatty acid content of the diet." (Emphasis added). Accordingly, Abayasekara et al. differentiates non-ruminants (swine are non-ruminants) from ruminants, and teaches away from increasing the supply of unsaturated fatty acids in the diets of non-ruminants because non-ruminants lack the particular microorganisms that cause extensive hydrogenation of fatty acids. Thus, the unsaturated fatty acids normally present in the diet of non-ruminants appear in the tissues so there is no reason to supplement the diet of non-ruminants with unsaturated fatty acids.

Furthermore, Abayasekara et al. provides no teaching that "C_{20} and C_{22} omega-3 fatty acids" have any particular effect on reproductive performance. The only disclosure in Abayasekara et al. directed to studies of the effects of "C_{20} and C_{22} omega-3 fatty acids," in particular, are on page 279 column 1, lines 4-36 and column 2, lines 1-28. Studies on the effects of "C_{20} and C_{22} omega-3 fatty acids" on prostaglandin and eicosanoid synthesis are described and, regarding prostaglandin synthesis, the authors of Abayasekara et al. conclude that "[t]he changes are not as expected" and, regarding eiconsanoid synthesis, the authors of Abayasekara et al. conclude that the effects are "hard to predict." Thus, page 279 column 1, lines 4-36 and column 2, lines 1-28 of Abayasekara et al. teaches that the effects of the C_{20} and C_{22} omega-3 fatty acids tested on prostaglandin and eicosanoid synthesis are not understood. Clearly, this disclosure in Abayasekara et al. cannot render obvious Appellants' claim 1.

Additional studies of the effects of PUFAs on ovulation, corpus luteum function, luteolysis, parturition, and lactation are described on pages 280-282 of Abayasekara et al., but the reference does not describe any studies using "C_{20} and C_{22} omega-3 fatty acids." Abayasekara et al. discloses studies to test the effects, on processes associated with reproduction, of "n-3 and n-6 PUFAs" in general, "fats," and "soybean oil and olive oil."

Soybean oil and olive oil contain the C_{18} omega-3 and omega-6 fatty acids, linoleic and linolenic acid, but do not contain "C_{20} and C_{22} omega-3 fatty acids." The effects of these lipids on processes associated with reproduction were variable and the authors of Abayasekara et al. conclude (page 282, last paragraph) that "[o]ur relative lack of knowledge means that it is impossible to predict at present whether particular dietary manipulations, which may be desirable from a human health viewpoint, will enhance or reduce fertility." Accordingly, because Abayasekara et al. teaches away from increasing the dietary supply of unsaturated fatty acids in non-ruminants, and provides no teaching of any effects of "C_{20} and C_{22} omega-3 fatty acids" on reproductive performance, the Examiner has not established a case of prima facie obviousness of claim 1 under 35 U.S.C. § 103(a) over Abayasekara et al.

On page 8, lines 13-16 of the February 26, 2004 office action, the Examiner makes the same misstatements regarding Appellants' alleged admissions as discussed above in section VIII. B. of this appeal brief. All of the same arguments discussed above in section VIII. B. of this appeal brief apply to the Examiner's discussion of Appellants' alleged admissions on page 8, lines 13-16 of the February 26 office action.

The Examiner also indicates with respect to optimization, that it would have been obvious to the skilled artisan to optimize the amount of the marine animal product, to achieve about 0.025% to about 2% by weight of the marine animal product in the feed composition, as specified in claim 1. This argument fails because Abayasekara et al. does not suggest using a marine animal product containing "C_{20} and C_{22} omega-3 fatty acids" in a feed composition for administration to female swine and, therefore, cannot suggest optimization of the amount of that marine animal product. Moreover, Abaysekara et al. is a general review paper which does not describe the specific composition of any of the diets disclosed in Abayasekara et al. or the specific protocols for administration of PUFAs to ruminants. Thus,

Abayasekara et al. does not provide any suggestion of optimizing to obtain a weight percentage of an oil from "about 0.025% to about 2%" in any feed composition.

Rejection of claims 2-6, 8-9, 13-20, 23, 25, 41, and 71-102

Each of claims 2-6, 8-9, 13-20, 23, 25, 41, and 71-102, requires that the feed composition used in the claimed method be administered to a "female swine" and each claim requires that the marine animal product in the feed composition used in the claimed method comprises "C_{20} and C_{22} omega-3 fatty acids," "C_{20} omega-3 fatty acids," or "C_{22} omega-3 fatty acids." Accordingly, all of the arguments discussed above for claim 1 apply with equal force to the rejection of claims 2-6, 8-9, 13-20, 23, 25, 41, and 71-102.

Moreover, Abaysekara et al. is a general review paper which does not describe the specific composition of any of the diets disclosed in Abayasekara et al. or the specific protocols for administration of PUFAs to ruminants. Accordingly, Abayasekara et al. does not teach, disclose, or suggest 1.) the limitations in claims 2, 9, 73, and 77 specifying that the marine animal product is a fish meal product, 2.) the limitations in claims 13-16, 78-81, 90-93, and 102 specifying regimens for administration of the feed composition to the female swine, 3.) the limitations in claims 3, 74, and 84 specifying that the marine animal product is an oil from a North Atlantic cold water fish, 4.) the limitations in claims 18 and 95 specifying that the fatty acids are stabilized by prilling, 5.) the limitations in claims 4, 8, 85, 86, and 88 specifying that the marine animal product is salmon oil, 6.) the limitations in claims 17, 82, and 94 specifying the composition further comprises an antioxidant, 7.) the limitations in claims 96-100 specifying that the marine animal product is menhaden oil, 8.) the limitations in claims 8, 9, 77, 86-89, 97, and 99-100 specifying a range of weight percentages of the marine animal product in the feed composition, or 9.) the limitations of claims 6, 76, and 83 specifying a range of omega-6 to omega-3 fatty acid ratios.

Appellants further point out that claims 14, 79, and 91 recite the step that the "feed composition is administered to the female swine beginning about 30 days before a first mating of the female swine during an estrus and continuing through a second mating of the female swine during the same estrus," which step is not taught or suggested in Abayasekara et al. Also, claim 102 recites the step that the "feed composition is fed to the female swine daily for the lifetime of the female swine," which step is not taught or suggested in Abayasekara et al. In addition claims 15, 80, and 92 recite that the "feed composition is administered to the female swine beginning about 1 to about 4 days prior to parturition and continuing through the next breeding," which step is not taught or suggested in Abayasekara et al.

With respect to the limitations of claims 6, 76, and 83 specifying a range of omega-6 to omega-3 fatty acid ratios, the Examiner indicates (see page 6, lines 14-16 of the February 26 office action) that Abayasekara et al. discloses these ratios in Fig. 1 on page 277 of Abayasekara et al. However, the designations in Fig. 1 on page 277 of Abayasekara et al. have nothing to do with omega-6 to omega-3 fatty acid ratios. The designations on page 277 of Abayasekara et al. are structural designations for fatty acids. For example, "22:6*n*-3" in reference to docosahexaenoic acid (DHA) means that DHA is a C_{22} omega-3 fatty acid with 6 double bonds (see page 7 of Appellants' specification for a description of the shorthand system for denoting the structure of fatty acids). Accordingly, for all of the reasons discussed above, the Examiner has not established a case of prima facie obviousness of claims 2-6, 8-9, 13-20, 23, 25, 41, and 71-102 under 35 U.S.C. § 103(a) over Abayasekara et al.

D. Appellants' claimed invention has met with great commercial success.

Although Appellants strongly contend that the Examiner has not established a prima facie case of obviousness under 35 U.S.C. § 103(a) with respect to Appellants' claims, a prima facie case of obviousness could be rebutted based on the great commercial success of Appellants' claimed invention. If a product that embodies the invention supplants prior art products and is a great commercial success, then it can be inferred that the invention was not obvious because otherwise persons lured by the prospect of success would have developed the invention sooner. *Pentec, Inc. v. Graphic Controls Corp.*, 776 F.2d 309, 227 U.S.P.Q. 766 (Fed. Cir. 1985); *Cable Electric Products, Inc. v. Genmark, Inc.*, 770 F.2d 1015, 226 U.S.P.Q. 881 (Fed. Cir. 1985). Appellants submitted the declaration of Dr. Donald E. Orr, under 37 C.F.R. § 1.132, with the response filed on October 21, 2002. In the declaration, Dr. Donald E. Orr, the President and Chief Operating Officer of United Feeds, the assignee of the captioned patent application, describes in detail the great commercial success of the product that is used in Appellants' claimed method. Appellants also submitted a supplemental declaration on October 21, 2003 to show that FERTILIUM™ meets the limitations of the claims. The commercial success of the product that is used in the claimed method establishes that the claimed invention is nonobvious.

As Dr. Orr asserts in the § 1.132 declaration, the product (FERTILIUM™) that embodies the claimed method was introduced into the marketplace in February of 2002. FERTILIUM™ is an animal feed additive that contains marine animal products, and is fed to swine to increase the reproductive performance of swine. As asserted by Dr. Orr, approximately 150,000 sows are already being fed FERTILIUM™, and current market analysis projections predict that FERTILIUM™ will be fed to approximately 600,000 sows by 2003 and 1,000,000 sows by 2004. Approximately 130,000 pounds of FERTILIUM™ are currently being ordered per month from United Feeds, and, based on current market analysis

projections, it is predicted that approximately 525,000 pounds per month will be ordered by 2003 and 875,000 pounds per month will be ordered by 2004.

As asserted in the § 1.132 declaration, the commercial acceptance of FERTILIUM™ is directly related to the claimed invention (*i.e.*, a method of increasing the reproductive performance of female swine by feeding the swine a composition comprising marine animal products). The swine production business is very competitive and margins are very close. Swine producers have been impressed by the consistently good results obtained (*i.e.*, increased reproductive performance in swine) when swine are fed FERTILIUM™. The increased reproductive performance in swine results from the marine animal products that are a component of FERTILIUM™. Thus, the effectiveness of FERTILIUM™ in increasing reproductive performance of swine, as a result of the marine animal products in FERTILIUM™, has led to the commercial success of the composition used in the claimed method.

The commercial success of FERTILIUM™ is evidenced in the § 1.132 declaration by the detailed sales and usage figures presented in the declaration, and, as Dr. Orr asserts in the declaration, the commercial success of FERTILIUM™ has been more rapid than expected based on his experience in new product development in the animal feed supplement market. Furthermore, as Dr. Orr asserts in the § 1.132 declaration, FERTILIUM™ already had three to five times the market share that a flaxseed-containing product (the alternative), that was on the market for a number of years before FERTILIUM™ was introduced, has for use in increasing reproductive performance in female swine, and, as asserted in the declaration, FERTILIUM™ had only been on the market for about 8 months. Therefore, the great commercial success of FERTILIUM™, the product that embodies the claimed method, and the short time within which FERTILIUM™ has supplanted prior art products in the same market (*i.e.*, the alternative flaxseed-containing product) indicate that

the claimed invention is nonobvious because otherwise people lured by the prospect of commercial success would have developed the claimed invention sooner.

In the February 26, 2004 office action, the Examiner indicates that the declaration of Dr. Donald E. Orr is insufficient to establish that Appellants' invention has met with great commercial success because the declaration merely shows the value of sales (in U.S. dollars) per the number of pounds sold in 2002 for FERTILIUM™ (see February 26 office action, page 10). The Examiner further indicates that a full market comparison with Appellants' competitors is required (see February 26 office action, page 10).

Contrary to the Examiner's argument, MPEP § 716.03(b) entitled "Commercial Success Derived From Claimed Invention" contains a section entitled "Sales Figures Must Be Adequately Defined." This section reads in its entirety as follows:

> Gross sales figures do not show commercial success *absent evidence as to market share, Cable Electric Products, Inc. v. Genmark, Inc.* 770 F.2d 1015, 226 USPQ 881 (Fed. Cir. 1985), or as to the time period during which the product was sold, or as to what sales would normally be expected in the market, *Es parte Standish*, 10 USPQ2d 1454 (Bd. Pat. App. & Inter. 1988).

(Emphasis Added). Thus, the MPEP indicates that gross sales figures do not show commercial success absent evidence as to market share, citing *Cable Electric Products, Inc. v. Genmark, Inc.* Alternatively, gross sales figures in combination with the time period during which the product was sold could be shown or gross sales figures in combination with evidence as to what sales would normally be expected in the market could be provided.

In *Cable Electric Products, Inc. v. Genmark, Inc.*, 226 USPQ at 888, the court discussed the issue of the Plaintiff's commercial success declaration as evidence of nonobviousness and indicated that the Plaintiff had only provided information as to number of units of its product, a night light, sold and the profit per night light. The court stated that "[w]hat it shows in relation to commercial success is fairly minimal. Without further economic evidence, for example, it would be improper to infer that the reported sales

represent a *substantial share of any definable market* or whether the profitability per unit is anything out of the ordinary in the industry involved." (Emphasis Added). *Id.* at 888. Thus, the court in *Cable Electric Products, Inc. v. Genmark, Inc.* and the MPEP indicate that gross sales figures in combination with evidence as to market share is sufficient to demonstrate commercial success.

In the § 1.132 declaration, filed on October 21, 2002, in response to the April 23, 2002 office action, Dr. Donald E. Orr states:

> FERTILIUM™ already has a market share that is three to five times greater than a flaxseed-containing product, and FERTILIUM™ has only been on the market for about 8 months. The flaxseed-containing product is an animal feed supplement containing ground flaxseed that is sold for use in increasing the reproductive performance of female swine, and the flaxseed-containing product had been on the market for a number of years before FERTILIUM™ was introduced to the marketplace. Therefore, FERTILIUM™, the product that embodies the claimed method, has met with great commercial success, and FERTILIUM™ has supplanted a flaxseed-containing product in the animal feed supplement market in the short time that FERTILIUM™ has been on the market.

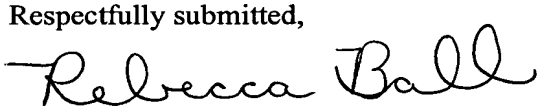
Accordingly, Appellants provided evidence in the declaration of Dr. Donald E. Orr of the market share that FERTILIUM™ has attained in the short time that it has been on the market, and Appellants provided evidence that FERTILIUM™ supplanted the flaxseed-containing product (the alternative) in the animal feed supplement market after only 8 months on the market. This evidence satisfies both the requirements of MPEP § 716.03(b), and the case law cited in the MPEP. The Examiner indicates that a full market comparison with Applicants' competitors is required (see February 26 office action, page 10), but, that is not what MPEP § 716.03(b) or the case law it cites requires, and it is unlikely that such a comparison could ever be done because it is doubtful that a competing business would divulge its monthly sales figures or its profitability to its competitor, especially when that competitor has supplanted it in the industry.

The Examiner also asserts (February 26 office action, pages 9-11) that no factual or documentary evidence has been provided in support of the composition of FERTILIUM™. Appellants submitted a supplemental declaration on October 21, 2003 describing the composition of FERTILIUM™ and showing that FERTILIUM™ meets the limitations of the claims. The Examiner also asserts that the declaration submitted fails to disclose the ingredients or agents in FERTILIUM™. The ingredients of FERTILIUM™ are disclosed in paragraphs 3 and 4 of the declaration. Thus, although Appellants contend that the Examiner has not established a prima facie case of obviousness under 35 U.S.C. § 103(a), based on the above arguments, any prima facie case of obviousness is overcome by the commercial success of Appellants' claimed invention.

CONCLUSION

Accordingly, Appellants submit that the Examiner's rejections of claims 1-6, 8-9, 13-20, 23, 25, 41, and 71-102 under 35 U.S.C. §103(a) are clearly erroneous. Appellants urge that the Board reverse the Examiner's 35 U.S.C. §103(a) rejections. Such action is respectfully requested.

Respectfully submitted,



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APPENDIX
CLAIMS ON APPEAL
U.S. APPLICATION SERIAL NO. 09/870,899

1. A method of increasing the reproductive performance of a female swine, comprising the step of administering to the female swine a feed composition comprising a marine animal product;

wherein the marine animal product comprises C_{20} and C_{22} omega-3 fatty acids or esters thereof; and

wherein the feed composition as a final mixture comprises about 0.025% to about 2% by weight of the marine animal product.

2. The method of claim 1 wherein the marine animal product is selected from the group consisting of a fish oil and a fish oil derived from a fish meal product or a mixture thereof.

3. The method of claim 1 wherein the marine animal product comprises a fish oil from a North Atlantic cold water fish.

4. The method of claim 3 wherein the fish oil comprises salmon oil.

5. The method of claim 1 wherein the feed composition further comprises omega-6 fatty acids or esters thereof.

6. The method of claim 5 wherein the omega-6 fatty acids/esters to omega-3 fatty acids/esters ratio in the feed composition as a final mixture is from about 3:1 to about 20:1.

7. (Canceled)

8. The method of claim 4 wherein the feed composition as a final mixture comprises about 0.025% to about 1% by weight of salmon oil.

9. The method of claim 2 wherein the feed composition as a final mixture comprises about 0.025% to about 1% by weight of the fish oil or the fish oil derived from the fish meal product.

10. (Canceled)

11. (Canceled)

12. (Canceled)

13. The method of claim 1 wherein the feed composition is administered daily to the female animal.

14. The method of claim 1 wherein the feed composition is administered to the female swine beginning about 30 days before a first mating of the female swine during an estrus and continuing through a second mating of the female swine during the same estrus.

15. The method of claim 1 wherein the feed composition is administered to the female swine beginning about 1 to about 4 days prior to parturition and continuing through the next breeding.

16. The method of claim 1 wherein the feed composition is administered during lactation.

17. The method of claim 1 wherein the feed composition as a final mixture further comprises an antioxidant.

18. The method of claim 1 wherein the omega fatty acids in the marine animal product are stabilized by prilling.

19. A method of increasing the number of live births to a female swine, comprising the step of administering to the female swine a feed composition comprising a marine animal product;

wherein the marine animal product comprises C_{20} and C_{22} omega-3 fatty acids or esters thereof; and

wherein the feed composition as a final mixture comprises about 0.025% to about 2% by weight of the marine animal product.

20. A method of increasing the total number of births to a female swine, comprising the step of administering to the female swine a feed composition comprising a marine animal product;

wherein the marine animal product comprises C_{20} and C_{22} omega-3 fatty acids or esters thereof; and

wherein the feed composition as a final mixture comprises about 0.025% to about 2% by weight of the marine animal product.

21. (Canceled)

22. (Canceled)

23. A method of increasing the uniformity of birth weight of offspring of a female swine, comprising the step of administering to the female animal a feed composition comprising a marine animal product;

wherein the marine animal product comprises C_{20} and C_{22} omega-3 fatty acids or esters thereof; and

wherein the feed composition as a final mixture comprises about 0.025% to about 2% by weight of the marine animal product.

24. (Canceled)

25. A method of increasing the farrowing rate of a female swine, comprising the step of administering to the female swine a feed composition comprising a marine animal product;

wherein the marine animal product comprises C_{20} and C_{22} omega-3 fatty acids or esters thereof; and

wherein the feed composition as a final mixture comprises about 0.025% to about 2% by weight of the marine animal product.

26. (Canceled)

27. (Canceled)

28. (Canceled)

29. (Canceled)

30. (Canceled)

31. (Canceled)

32. (Canceled)

33. (Canceled)

34. (Canceled)

35. (Canceled)

36. (Canceled)

37. (Canceled)

38. (Canceled)

39. (Canceled)

40. (Canceled)

41. A method of increasing the reproductive performance of a breeding population of swine comprising the step of:

administering to a female swine a feed composition comprising a marine animal product;

wherein the marine animal product comprises C_{20} and C_{22} omega-3 fatty acids or esters thereof; and

wherein the feed composition as a final mixture comprises about 0.025% to about 2% by weight of the marine animal product.

42. (Canceled)

43. (Canceled)

44. (Canceled)

45. (Canceled)

46. (Canceled)

47. (Canceled)

48. (Canceled)

49. (Canceled)

50. (Canceled)

51. (Canceled)

52. (Canceled)

53. (Canceled)

54. (Canceled)

55. (Canceled)

56. (Canceled)

57. (Canceled)

58. (Canceled)

59. (Canceled)

60. (Canceled)

61. (Canceled)

62. (Canceled)

63. (Canceled)

64. (Canceled)

65. (Canceled)

66. (Canceled)

67. (Canceled)

68. (Canceled)

69. (Canceled)

70. (Canceled)

71. A method of increasing the reproductive performance of a female swine, comprising the step of administering to the female swine a feed composition comprising a marine animal product;

wherein the marine animal product comprises C_{20} omega-3 fatty acids or esters thereof; and

wherein the feed composition as a final mixture comprises about 0.025% to about 2% by weight of the marine animal product.

72. A method of increasing the reproductive performance of a female swine, comprising the step of administering to the female swine a feed composition comprising a marine animal product;

wherein the marine animal product comprises C_{22} omega-3 fatty acids or esters thereof; and

wherein the feed composition as a final mixture comprises about 0.025% to about 2% by weight of the marine animal product.

73. A method of increasing the reproductive performance of a female swine, comprising the step of administering to the female swine a feed composition comprising a marine animal product wherein the marine animal product is a fish meal product and wherein the fish meal product comprises C_{20} and C_{22} omega-3 fatty acids or esters thereof.

74. The method of claim 73 wherein the fish meal product is from a North Atlantic cold water fish.

75. The method of claim 73 wherein the feed composition further comprises omega-6 fatty acids or esters thereof.

76. The method of claim 75 wherein the omega-6 fatty acids/esters to omega-3 fatty acids/esters ratio in the feed composition as a final mixture is from about 3:1 to about 20:1.

77. The method of claim 73 wherein the feed composition as a final mixture comprises about 1% to about 10% by weight of the fish meal product.

78. The method of claim 73 wherein the feed composition is administered daily to the female animal.

79. The method of claim 73 wherein the feed composition is administered to the female swine beginning about 30 days before a first mating of the female swine during an estrus and continuing through a second mating of the female swine during the same estrus.

80. The method of claim 73 wherein the feed composition is administered to the female swine beginning about 1 to about 4 days prior to parturition and continuing through the next breeding.

81. The method of claim 73 wherein the feed composition is administered during lactation.

82. The method of claim 73 wherein the feed composition as a final mixture further comprises an antioxidant.

83. A method of increasing the reproductive performance of a female swine, comprising the step of administering to the female swine a feed composition comprising a marine animal product;

wherein the marine animal product comprises omega-6 fatty acids or esters thereof and C_{20} and C_{22} omega-3 fatty acids or esters thereof; and

wherein the omega-6 fatty acids/esters to omega-3 fatty acids/esters ratio in the feed composition as a final mixture is from about 3:1 to about 20:1.

84. The method of claim 83 wherein the marine animal product is an oil from a North Atlantic cold water fish.

85. The method of claim 83 wherein the marine animal product comprises salmon oil.

86. The method of claim 85 wherein the feed composition as a final mixture comprises about 0.025% to about 1% by weight of salmon oil.

87. The method of claim 83 wherein the feed composition as a final mixture comprises about 0.025% to about 1% by weight of the marine animal product.

88. The method of claim 85 wherein the feed composition as a final mixture comprises about 0.025% to about 2% by weight of salmon oil.

89. The method of claim 83 wherein the feed composition as a final mixture comprises about 0.025% to about 2% by weight of the marine animal product.

90. The method of claim 83 wherein the feed composition is administered daily to the female animal.

91. The method of claim 83 wherein the feed composition is administered to the female swine beginning about 30 days before a first mating of the female swine during an estrus and continuing through a second mating of the female swine during the same estrus.

92. The method of claim 83 wherein the feed composition is administered to the female swine beginning about 1 to about 4 days prior to parturition and continuing through the next breeding.

93. The method of claim 83 wherein the feed composition is administered during lactation.

94. The method of claim 83 wherein the feed composition as a final mixture further comprises an antioxidant.

95. The method of claim 83 wherein the omega fatty acids in the marine animal product are stabilized by prilling.

96. The method of claim 1 wherein the marine animal product is menhaden oil.

97. The method of claim 96 wherein the feed composition as a final mixture comprises about 0.025% to about 1% by weight of menhaden oil.

98. The method of claim 83 wherein the marine animal product is menhaden oil.

99. The method of claim 98 wherein the feed composition as a final mixture comprises about 0.025% to about 1% by weight of menhaden oil.

100. The method of claim 98 wherein the feed composition as a final mixture comprises about 0.025% to about 2% by weight of the menhaden oil.

101. The method of claim 1 wherein the feed composition further comprises a plant oil.

102. The method of claim 1 wherein the feed composition is fed to the female swine daily for the lifetime of the female swine.

Enrichment of Omega-3 Fatty Acids in Suckling Pigs by Maternal Dietary Fish Oil Supplementation[1,2,3]

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ABSTRACT: This study was designed to determine whether substituting menhaden fish oil (FO) for lard (LA) in a practical sow diet was a suitable method for enriching newborn pigs with omega-3 polyunsaturated fatty acids (*n*-3 PUFA). On d 107 of gestation, 18 crossbred sows were randomly allotted to one of three experimental diets, in which FO was substituted for LA at 0, 3.5, and 7% of the diet. On d 1, 7, 14, and 21 after farrowing samples of milk and serum from the sows and pig serum were collected for fatty acid analysis. The content of *n*-3 PUFA in the serum of sows fed FO increased six-fold over that in serum of LA-fed sows ($P < .0001$). Feeding FO decreased the levels of arachidonic acid in maternal serum by approximately 50% ($P < .0001$). Similar changes were reflected in the fatty acid profiles of sow's milk. Pig serum *n*-3 PUFA levels were elevated over 5- and 10-fold within 24 h of birth in those litters born to sows fed 3.5 and 7% fish oil, respectively. Eicosapentaenoic acid levels in pig serum increased linearly ($P < .01$) during the first 2 wk postnatally in pigs suckling FO-fed sows and accounted for as much as 12% of the total fatty acids present on d 21. In conclusion, we have demonstrated that feeding FO to sows during late gestation and lactation enriches the newborn pig with *n*-3 PUFA.

Key Words: Omega-3 Fatty Acids, Milk, Serum, Sows, Pigs

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Introduction

The health of pigs has a great effect on efficiency of pork production. Various efforts, including dietary treatment, have been attempted to reduce moribundity and mortality of newborn pigs. The concept of manipulating the maternal diet to alter milk composition in order to benefit the offspring is not new (Moser, 1985; Farnworth and Kramer, 1987). Numerous investigators have explored the effect of changing milk fat composition on sow performance and pig survival (Witter and Rook, 1970; Seerley et al., 1974). However, evidence that supplementing a sow's diet with fat benefits the nursing pigs is equivocal (Moser, 1985).

Dietary *n*-3 polyunsaturated fatty acids (PUFA) have been reported to affect heart disease, autoimmune disease, and inflammatory diseases in humans and laboratory animals (Simopoulos et al., 1986). However, little attention has been given to the possible beneficial effects of *n*-3 PUFA-rich oils, such as fish oils, in domestic animal production. The primary objective of this study was to determine whether neonatal pigs could be supplied with *n*-3 PUFA by feeding a source of these fatty acids to the sow.

Materials and Methods

Animals and Diets

On d 107 of gestation, 18 crossbred sows (Landrace × Duroc) were randomly allotted to one of three experimental diets, in which menhaden fish oil was substituted for lard at 0, 3.5, and 7% of the diet (Table 1, treatment groups **LA**, **MIX**, **FO**, respectively). Diets were isoenergetic and were formulated to contain 7% added fat and to meet National Research Council requirements for the lactating sow (NRC, 1988). Sows were individually fed 1.8 to 2.1 kg of experimental diets once daily from d 107 of gestation until farrowing. An additional .45 kg was fed per day for each suckling pig during the 28-d lactation period. No attempt was made to restrict newborn pigs from eating the experimental diets. Feed consumption and

[1]Appreciation is expressed to Zapata Haynie Inc., Reedville, VA, for supplying the menhaden oil used; to Lyndle Vanskike for assistance with the care and feeding of the research animals; and to Mark Ellersieck for assistance with the statistical analyses.

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Table 1 Composition of sow diets

Ingredient	Dietary treatment groups[a]		
	LA	MIX	FO
	%		
Ground corn	58.9	58.9	58.9
Oats	10.1	10.1	10.1
Soybean meal (44% CP)	20.0	20.0	20.0
Lard[b]	7.0	3.5	—
Fish oil[c]	—	3.5	7.0
Dicalcium phosphate	2.7	2.7	2.7
Vitamin mix[d]	.5	.5	.5
Trace mineral salt mix[e]	.5	.5	.5
Ground limestone	.4	.4	.4

[a]Designation of experimental diets, in which fish oil is substituted for lard at 0, 3.5, and 7% of the diet (LA, MIX, and FO groups, respectively).

[b]Analyzed fatty acid composition of (percentage of weight): C14:0, 1.0; C16:0, 25.4; C16:1*n*-7, 2.8; C18:0, 13.7; C18:1*n*-9, 42.3; C18:2*n*-6, 10.8; C18:3*n*-3, .7.

[c]Menhaden fish oil courtesy of Zapata Haynie Corp. (Reedville, VA) with an analyzed fatty acid composition of (percentage of weight): C14:0, 6.4; C16:0, 16.6; C16:1*n*-7, 10.4; C18:0, 2.6; C18:1*n*-9, 13.9; C18:2*n*-6, 1.5; C18:3*n*-3, 1.2; C20:1*n*-9 and *n*-11, 2.6; C20:5*n*-3, 14.3; C22:1*n*-11, 1.8; C22:5*n*-3, 2.2; C22:6*n*-3, 12.6. The fish oil was stabilized against autooxidation by the addition of 500 ppm of ethoxyquin.

[d]Supplied per kilogram of diet: 5,506 IU of vitamin A, 550 IU of vitamin D_3, 27 IU of vitamin E, 5.5 mg of riboflavin, 27 mg of d-pantothenic acid, 33 mg of niacin, 2.2 mg of thiamin, 2.2 mg of vitamin B_6, 1.1 mg of folic acid, 669 mg of choline, .038 mg of vitamin B_{12} 2.2 mg of menadione, and .165 mg of d-biotin.

[e]Supplied in milligrams per kilograms of diet: 125 of Zn, 125 of Fe, 25 of Mn, 15 of Cu, .5 of I, .1 of Se, and 4,258 of NaCl.

changes in BW for each sow were recorded, as were the number of pigs born per sow, birth weight, and weaning weights. Litters were adjusted in size to 8 to 10 pigs per litter. Pigs were transferred within treatment groups, or in the case of the MIX group, some pigs were moved to sows not in the study. Three sows were removed from the study before farrowing (one from LA and two from MIX) for medical reasons unrelated to the dietary treatments.

This study was conducted during the winter of 1989 in a fully enclosed gestation and farrowing building described previously (Hamilton and Veum, 1984). The farrowing rooms were thermostatically maintained at a minimum temperature of 20°C. Radiant heaters provided supplemental heat for the baby pigs. Housing, handling, and sample collection procedures conformed to policies and recommendations of the University of Missouri's Laboratory Animal Care Advisory Committee.

Sample Collection

Before allotment to diet groups, samples of blood from each sow were collected. On d 1, 7, 14, and 21 after farrowing samples of colostrum or milk, sow serum, and pig serum were collected. Approximately 50 mL of colostrum or milk was obtained by milking several udders after the young pigs had been removed from the sow for ≥ 1 h. Milk let-down was induced by an i.m. injection of 10 IU of oxytocin (Ferment Animal Health, Kansas City, MO). Samples from randomly selected glands were pooled, placed on ice, and transported to the laboratory, where a portion of each sample was frozen for later analysis. Blood (5 to 10 mL), collected from the jugular vein, was allowed to clot at room temperature. Serum was collected after centrifugation (900 × *g* for 15 min). Serum samples were stored at −20°C until they were extracted for fatty acid analysis.

Lipid Extraction and Fatty Acid Analysis

Samples of colostrum, milk, or serum (.5 mL) were diluted 1:1 with Tris/EDTA/sucrose buffer (50 m*M* Trizma-HCl; 1 m*M* EDTA; .32 *M* sucrose; pH 7.4). Lipids were extracted as described by Folch et al. (1957) with minor modifications. Briefly, 1 mL of the diluted samples was mixed with 4 mL of chloroform and methanol (2:1, vol/vol). The organic phase containing the lipid extract was removed and the aqueous phase was re-extracted with 4 mL of chloroform/methanol/12 *N* HCl (2:1:.013, by vol). The acidified organic layer was neutralized with one drop of concentrated NH_4OH, then the organic layers were pooled, filtered over Na_2SO_4, and reduced in volume under nitrogen. The percentage of lipid in the milk samples was determined gravimetrically.

Dietary fats and total lipids extracted from samples were transmethylated with 4 mL of 4% sulfuric acid in methanol for 1 h in a 88°C heating block. The mixture was saponified according to methods described by Kates (1986) by the addition of 33% potassium hydroxide and subsequent heating at 88°C for 1 h. Two washes of heptane were used to remove nonsaponifiable materials. The solution was acidified by the addition of 1 mL of 6 *N* hydrochloric acid. The free fatty acids were extracted twice with 5 mL of heptane. The free fatty acids were remethylated as described above. The fatty acid methyl esters were extracted by adding 4 mL of water and 5 mL of heptane to the test tube. The top heptane layer was transferred to sample tubes and evaporated to dryness under a stream of nitrogen. The samples were redissolved in 100 μL of heptane.

Fatty acid methyl esters (**FAME**) were analyzed using a Hewlett-Packard gas-liquid chromatograph, Model 5890 (Sunnyvale, CA) equipped with a 30 m × .25 mm i.d. fused silica capillary column (SUPELCOWAX 10, Supelco, Bellefonte, PA). Helium was the carrier gas, and the flow rate was set at 1 mL/min. A timed temperature program was used; the initial oven temperature was 190°C (10 min), then this was raised 3C°/min to 230°C and held for 14 min. The FAME were identified by comparing relative retention times of commercially available standards (PUFA-1 and PUFA-2; Supelco). Results, expressed as percentage of total fatty acids, were determined using a Hewlett-Packard 3390 integrator.

Statistical Methods

The data were analyzed as a split-plot in time for all data. The linear statistical model contained the effect of diet, animal(diet), time, and the interaction of diet × time. Diet × time interactions were evaluated for sow data for samples taken after the treatments were imposed. The pig data used the litter as the experimental unit; thus, the main plot error was litter(diet), instead of animal(diet) (Gill and Hafs, 1971; SAS, 1985). Mean differences were ascertained using Fisher's LSD test as it pertains to a split-plot design (Steel and Torrie, 1980). Differences among diet groups were determined by computing linear, quadratic, and cubic contrasts and tested using a 1 df *F*-test (Carmer and Seif, 1963). The sum of squares for differences among dietary treatments were calculated as outlined by Snedecor and Cochran (1967). A 1 df *F*-value was calculated to determine significance. The main plot effect of diet was tested using the main plot error as the denominator of the *F*-value. Time, the interaction of diet × time, and all trend contrasts were tested using the residual error term.

Results and Discussion

Litter Size, Birth Weights, and Weaning Weights

There were no significant differences in feed consumption or weight loss by sows among treatment groups (data not shown). Similarly, the number of live pigs born per litter (9.4, 11.8, and 8.2 ± 1.2; P = .29; n = 5, 4, and 6, for LA, MIX, and FO groups, respectively) and pig birth weights (1.51, 1.42, and 1.50 ± .05 kg; P = .25, for LA, MIX, and FO groups, respectively) did not differ among treatment groups. Weaning weights for the MIX group were lower (P < .007) than those for the other groups (13.5 vs 16.8 and 16.0 ± .7 kg for MIX, LA, and FO, respectively). However, differences in age at weaning (28.8, 25.6, and 26.8 ± .33 d) accounted for most of this disparity in weaning weights between diet groups. When these data were analyzed with age of weaning as a covariate, no diet effect was noted (P > .05). This is contrary to the observations of Yeh et al. (1990), who reported a 5 to 10% greater weight gain in the rats suckling dams fed fish oil vs corn oil. The limited number of litters evaluated in our study does not allow us to evaluate adequately the effect on pigs' weaning weights of supplementing the sows' diets with fish oil.

Serum Fatty Acid Profiles of Sows

The substitution of fish oil for lard in sows' diets altered the fatty acid profile of the sows' serum in a time- and concentration-dependent manner (Table 2). The most prominent effects observed were an increase in *n*-3 PUFA and a corresponding decrease in the *n*-6 PUFA. The net result was that total PUFA levels were similar (approximately 50%) across all treatment groups. The overall percentage of saturated, monounsaturated, and PUFA in the serum changed little, even though PUFA levels in the diet differed by as much as twofold (22 vs 41% total PUFA in the LA and FO diets, respectively). However, the inclusion of fish oil increased (P < .01) the overall content of *n*-3 PUFA from < 4% to 13 and 23% of the total fatty acids present, depending on the amount of fish oil in the diet (3.5 or 7%, respectively).

The inclusion of fish oil in the diet elevated sow serum eicosapentaenoic acid (**EPA**, 20:5*n*-3) levels linearly (P < .01) and quadratically (P < .01) over time (Figure 1). This effect was rapid: more than one-half of the increase occurred within the 1st wk of

Table 2. Average weight percentage of total serum fatty acids of sows fed lard (LA), menhaden fish oil (FO), or a 1:1 mixture of both fat sources (MIX)[a]

Fatty acid[b]	Treatment			SEM
	LA	MIX	FO	
	% wt/wt[c]			
14:0	.5[f]	.8[g]	1.2[g]	.2
16:0	14.6[f]	18.4[g]	15.3[f]	.8
16:1	2.5[f]	2.5[f]	3.9[g]	.2
18:0	8.4	9.5	8.0	.7
18:1	22.1[f]	22.9[f]	16.9[g]	1.5
18:2*n*-6	30.6[f]	24.4[g]	20.3[h]	1.4
20:4*n*-6	12.6[f]	5.7[g]	5.7[g]	.9
20:5*n*-3	.6[f]	7.9[g]	16.2[h]	1.1
22:5*n*-3	1.1[f]	1.7[g]	1.7[g]	.1
22:6*n*-3	.8[f]	2.8[g]	4.2[h]	.3
SAT[d]	23.5[f]	28.6[g]	24.5[f]	1.3
MONO[d]	24.8[fg]	25.5[f]	21.0[g]	1.3
PUFA[d]	49.3	44.0	50.8	2.4
Total *n*-6[e]	46.0[f]	31.0[g]	27.8[g]	2.1
Total *n*-3[e]	3.3[f]	13.0[g]	23.0[h]	1.4

[a]On d 107 d of gestation sows were fed experimental diets in which fish oil was substituted for lard at 0, 3.5, and 7% of the diet (LA, MIX, and FO groups, respectively). Serum samples were collected from each sow before their allotment to treatment groups, as well as on d 1, 7, 14, and 21 after farrowing. Lipids were extracted from serum and analyzed by gas-liquid chromatography as described in the Materials and Methods section.

[b]Fatty acids are denoted by the number of carbons: number of double bonds, followed by the position of the first double bond relative to the methyl-end (*n*-).

[c]Values represent the percentage of total fatty acids and are expressed as least squares means across all sampling times; means with a superscript that do not have a common superscript letter (f, g, and h) differ (P < .05). Only fatty acids that accounted for ≥ 1% of the total are presented. The following fatty acids had a significant diet × time interaction: 18:0, 20:4*n*-6, and 20:5*n*-3. For these latter two fatty acids this interaction is illustrated in Figures 1 and 2. The time-dependent changes in 18:0 levels were small (2 to 4%) within treatment groups and are not shown.

[d]SAT = sum total area percentage of 14:0, 16:0, 18:0; MONO = sum total area percentage of 16:1*n*-7, 18:1*n*-7, 18:1*n*-9, and 20:1*n*-9; PUFA = sum total area percentage of 18:2*n*-6, 18:3*n*-6, 18:3*n*-3, 20:2*n*-6, 20:3*n*-6, 20:4*n*-6, 20:5*n*-3, 22:4*n*-6, 22:5*n*-6, 22:5*n*-3, and 22:6*n*-3.

[e]Total *n*-6 = sum total area percentage of 18:2*n*-6, 18:3*n*-6, 20:2*n*-6, 20:3*n*-6, 20:4*n*-6, 22:4*n*-6, and 22:5*n*-6; total *n*-3 = sum total area percentage of 18:3*n*-3, 20:5*n*-3, 22:5*n*-3, and 22:6*n*-3.

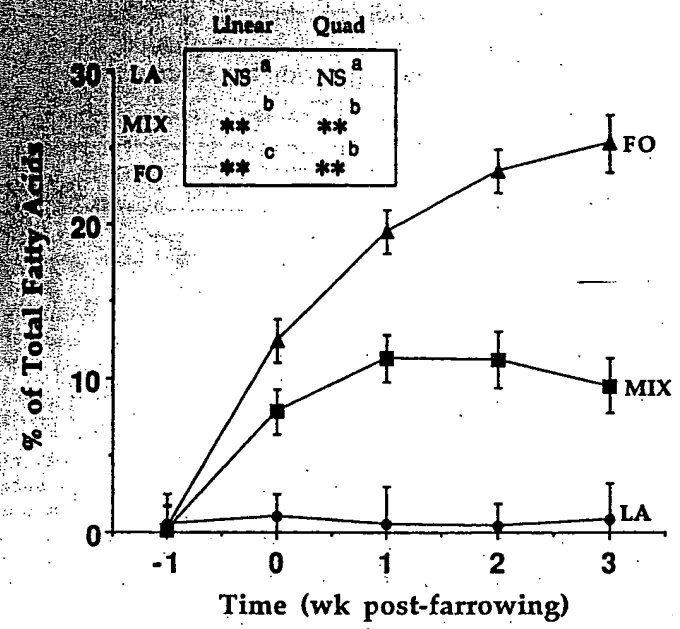

Figure 1. Effect of feeding fish oil on sows' serum eicosapentaenoic acid (EPA) levels over time. Values represent the amount of EPA (20:5*n*-3) as a percentage of total fatty acids present and are expressed as least squares means. Error bars represent SEM. Results from orthogonal analysis are depicted in the inset box (Carmer and Seif, 1963). Single and double asterisks represent significant linear or quadratic trends within a diet treatment with $P < .05$ and .01, respectively; NS represents $P > .05$. Differences among diet treatment groups, but within linear or quadratic polynomials, are depicted with different superscripts ($P > .05$; Snedecor and Cochran, 1967). Values for sows fed lard (LA), fish oil (FO), and the 1:1 mix of lard and fish oil (MIX) were significantly different ($P < .05$) from each other at all time points, except at −1 wk. The following regression equations best fit the FO and MIX data, respectively: $Y_{(FO)} = -14.16 + 16.373x - 1.7071x^2$, $R^2 = .998$; $Y_{(MIX)} = -9.88 + 11.82x - 1.6x^2$, $R^2 = .992$.

feeding fish oil. Furthermore, the rate of increase was greater ($P < .05$) for the sows fed 7 vs 3.5% fish oil. There was a direct relationship between the level of fish oil in the diet and the increase in EPA levels in the serum.

This dose-response relationship held for docosahexaenoic acid (**DHA**, 22:6*n*-3), but not for docosapentaenoic acid (**DPA**, 22:5*n*-3). Furthermore, the magnitude of the response seemed to be proportional to the concentration of *n*-3 PUFA in the fish oil (i.e., EPA > DHA > DPA). Initial levels of DHA were < 1% of the total serum fatty acids. This level of DHA is approximately 50 to 75% lower than the values typically reported in humans and rodents. These low levels may reflect a lower intake of *n*-3 PUFA by the sows.

Arachidonic acid (**AA**, 20:4*n*-6) accounted for 10 to 12% of the total fatty acids present in the sows' serum at the start of the trial. When fish oil was added to the diet, AA levels in the serum declined linearly ($P < .01$) over time to < 5% of the total fatty acids present within 2 wk (Figure 2). Unlike its effect on EPA, the effect of feeding fish oil on AA was similar at both levels of fish oil.

Milk Fatty Acid Profiles

Feeding fish oil changed the fatty acid composition of sows' milk (Table 3) and modestly increased ($P < .05$) the PUFA content of the milk. The long-chain PUFA (C20 or greater) content of milk from LA-fed sows was 2.9%, compared with 6.9 and 9.7% for milk from sows fed 3.5 and 7% fish oil, respectively. However, the actual amount of lipid in the milk (9.4% wet weight basis) was not affected by the dietary treatments. The milk fat content was similar to that reported in other studies in which sows' diets were supplemented with fat (Moser, 1985). Furthermore, the fatty acid profile of the milk from lard-fed sows was similar to that reported by others (DeMan and Bowland, 1963; Arbuckle et al., 1991).

Levels of EPA in the milk were elevated as much as sixfold by fish oil ($P < .0001$). Similar results have been reported for a variety of other species, including humans (Harris et al., 1984) and rats (Yeh et al., 1990). However, unlike in the sows' serum, milk EPA remained a relatively minor constituent, accounting for slightly more than 2 and 3% of the total fatty acids in sows fed 3.5 vs 7% fish oil, respectively. The enrichment of EPA in sows' milk was intermediate to that reported for rats and humans. Yeh et al. (1990) reported that EPA levels in rats' milk increased from < .1 to > 10% of total fatty acids. Harris et al. (1984) reported that the EPA content of human milk was elevated from trace levels up to .3 to .5% of total fatty acids with fish oil supplementation. In our estimation, the total *n*-3 PUFA intake for the women, sows, and rat dams used in each of these studies were as follows: .05, .23, and 2.0 $g \cdot kg^{-1} \cdot d^{-1}$, respectively. Thus, the differences in the magnitude of this response across the species may in part be a consequence of differences in the amount of EPA consumed.

In this study, the relative levels of *n*-3 fatty acids in the milk remained constant throughout lactation ($P < .74$ for the time effect). In the study with humans, *n*-3 PUFA content of the milk reached a plateau after 1 to 2 wk (Harris et al., 1984). However, fish oil supplementation was initiated 3.5 mo into lactation, on average. Furthermore, the rate of change was dependent on the dose of fish oil fed. Apparently, in our study, the 1 wk of FO feeding before farrowing was long enough to modify the fatty acid composition of the mammary tissues and blood lipids such that no further alterations in milk fatty acid profiles were evident.

Colostrum had a higher percentage of PUFA (22.6 vs 18.7%) and a lower level of saturated fatty acids (29.8 vs 35.6%) than mature milk ($P < .0001$). Most

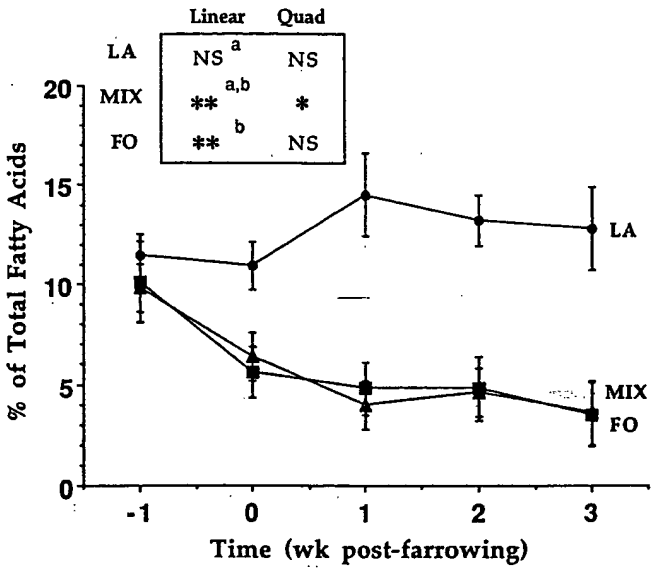

Figure 2. Effect of feeding fish oil on sows' serum arachidonic acid (20:4*n*-6) levels over time. Conditions and data analysis were as described in Figure 1. Values for sows fed fish oil (FO) and the 1:1 mix of lard and fish oil (MIX) were significantly different ($P < .05$) from those for the sows (LA) fed lard at all time points, except at −1 wk. The following regression equations best fit the FO and MIX data, respectively: $y_{(FO)} = 9.94 - 1.42x$, $R^2 = .781$; $y_{(MIX)} = 13.56 - 4.4857x + .51429x^2$, $R^2 = .903$.

of this difference was related to the higher levels of linoleic acid (18:2*n*-6) in colostrum vs milk (15.5 vs 11.2%, respectively). The level of *n*-3 PUFA in colostrum was similar to that in mature milk.

Serum Fatty Acid Profiles of Pigs

Feeding fish oil to sows significantly modified the serum fatty acid profile of pigs born to, and suckling, those sows (Table 4). Differences in the total saturated and monounsaturated fatty acids ($P < .05$) were noted in the pigs' serum from different treatment groups. These changes were modest compared with the alterations in most of the long-chain PUFA. Serum *n*-3 PUFA levels were elevated in pigs suckling sows fed fish oil ($P < .0005$). Initial EPA levels in the serum (from samples collected within 24 h of birth) were significantly higher in MIX and FO pigs than in LA pigs (5.2 and 7.3 vs 0.4% of the total fatty acids present, respectively). It is not clear how much of this elevation in *n*-3 PUFA is a result of placental transfer. Placental transfer of fatty acids in swine has been reported to be quite limited (Thulin et al., 1989; Ramsay et al., 1991). However, in a subsequent study we observed an elevation in *n*-3 PUFA in pigs born to sows fed fish oil before providing them access to the sow (total *n*-3 PUFA of 16.6 ± 2.0 vs 5.8 ± .9% for FO and LA pigs, respectively; $P < .005$; n = 4).

Table 3. Average weight percentage of fatty acids from the milk of sows fed lard (LA), menhaden fish oil (F), or a 1:1 mixture of both fat sources (MIX)[a]

	Treatment			
Fatty acids[b]	LA	MIX	FO	SEM
	% wt/wt[c]			
14:0	2.9	3.4	3.4	.3
16:0	26.8	25.9	25.6	1.1
16:1	8.0	8.0	8.1	.5
18:0	4.6	4.8	5.1	.2
18:1	39.2	32.4	32.0	2.5
18:2*n*-6	13.1	12.6	11.3	.5
18:3*n*-3	.6[g]	.8[fg]	.9[f]	.1
20:4*n*-6	.7[f]	.5[g]	.7[f]	.1
20:5*n*-3	.5[g]	2.3[f]	3.3[f]	.5
22:5*n*-3	.4[g]	1.1[f]	1.3[f]	.2
22:6*n*-3	.6[g]	2.4[f]	3.5[f]	.5
SAT[d]	34.2	34.0	34.0	1.3
MONO[d]	47.3[g]	41.1[f]	40.3[f]	2.1
PUFA[d]	16.6[f]	20.3[fg]	22.1[g]	1.2
Total *n*-6[e]	14.7	13.8	13.0	.6
Total *n*-3[e]	2.1[g]	6.6[f]	9.1[f]	1.2

[a]On d 107 of gestation sows were fed experimental diets in which fish oil was substituted for lard at 0, 3.5, and 7% of the diet (LA, MIX, and FO groups, respectively). After farrowing, milk samples were collected at the same time periods described for the sows' serum (i.e., d 1, 7, 14, and 21 after farrowing). Total milk lipids were extracted and analyzed by gas-liquid chromatography as described in the Materials and Methods section. All variables in this table showed no significant interaction effect of diet × time; therefore, the main effect means are presented. Means within rows lacking a common superscript letter (f and g) differ ($P < .05$). Only fatty acids that accounted for ≥ .5% of the total are presented.

[b]Fatty acids are denoted by the number of carbons: number of double bonds, followed by the position of the first double bond relative to the methyl-end (*n*-).

[c]Values represent the percentage of total fatty acids and are expressed as least squares means across all sampling times; means with a superscript that do not have a common superscript letter (f, g, and h) differ ($P < .05$). Only fatty acids that accounted for ≥ 1% of the total are presented. The following fatty acids had a significant diet × time interaction: 18:0, 20:4*n*-6, and 20:5*n*-3. For these latter two fatty acids this interaction is illustrated in Figures 1 and 2. The time-dependent changes in 18:0 levels were small (2 to 4%) within treatment groups and are not shown.

[d]SAT = sum total area percentage of 14:0, 16:0, 18:0; MONO = sum total area percentage of 16:1*n*-7, 18:1*n*-7, 18:1*n*-9, and 20:1*n*-9; PUFA = sum total area percentage of 18:2*n*-6, 18:3*n*-6, 18:3*n*-3, 20:2*n*-6, 20:3*n*-6, 20:4*n*-6, 20:5*n*-3, 22:4*n*-6, 22:5*n*-6, 22:5*n*-3, and 22:6*n*-3.

[e]Total *n*-6 = sum total area percentage of 18:2*n*-6, 18:3*n*-6, 20:2*n*-6, 20:3*n*-6, 20:4*n*-6, 22:4*n*-6, and 22:5*n*-6; total *n*-3 = sum total area percentage of 18:3*n*-3, 20:5*n*-3, 22:5*n*-3, and 22:6*n*-3.

The EPA levels increased linearly with age ($P < .01$) in MIX and FO pigs and quadratically ($P < .01$) in FO pigs (Figure 3). As observed in the sow, the rate of increase and the final EPA levels in pigs' serum was greater in the FO than in the MIX pigs ($P < .05$). The increase in *n*-3 PUFA over time in the pigs' serum demonstrates that the neonatal pig efficiently digests and absorbs *n*-3 PUFA fatty acids derived from the milk. This agrees with the work of Chiang et al. (1989), who that showed that neonatal pigs utilized fish oil at a 99% efficiency. The level of AA in the serum was lower in the pig than in the sow

Table 4. Average weight percentage of total serum fatty acids of pigs suckling sows fed lard (LA), menhaden fish oil (FO), or a 1:1 mixture of both fat sources (MIX)[a]

Fatty acid[b]	Treatment LA	MIX	FO	SEM
	% wt/wt[c]			
14:0	1.1	1.1	1.2	.3
16:0	19.9[f]	23.5[g]	20.2[fg]	1.2
16:1	5.8	4.6	4.2	.6
18:0	7.0	8.6	8.3	.8
18:1	23.8[f]	20.1[fg]	17.7[g]	1.5
18:2*n*-6	23.0[f]	19.1[g]	17.1[g]	.9
18:3*n*-6	.6[f]	.3[g]	.3[g]	.1
18:3*n*-3	.5	.5	.5	.1
20:4*n*-6	8.7[f]	4.3[g]	4.9[g]	.5
20:5*n*-3	.3[f]	7.6[g]	12.4[h]	.8
22:5*n*-3	.7[f]	1.5[g]	1.4[g]	.2
22:6*n*-3	1.2[f]	4.6[g]	5.6[g]	.5
SAT[d]	28.0[f]	33.2[g]	29.6[f]	2.4
MONO[d]	30.0[f]	24.9[g]	22.0[g]	3.4
PUFA[d]	36.8[f]	38.4[f]	42.9[g]	2.9
Total *n*-6[e]	34.1[f]	24.3[g]	23.0[g]	1.2
Total *n*-3[e]	2.7[f]	14.3[g]	19.9[h]	1.2

[a]On d 107 of gestation sows were fed experimental diets in which fish oil was substituted for lard at 0, 3.5, 7% of the diet (LA, MIX, and FO groups, respectively). After farrowing, serum samples were collected at the same time periods described for the sows' serum (i.e., d 1, 7, 14, 21 after farrowing). Total serum lipids were extracted and analyzed by gas-liquid chromatography as described in the Materials and Methods section. No variables in this table showed a significant interaction effect of diet × time; so the main effect means are presented. Means within rows lacking a common superscript letter (f, g, h) differ ($P < .05$). Only fatty acids that accounted for ≥ .5% of the total are presented.

[b]Fatty acids are denoted by the number of carbons: number of double bonds, followed by the position of the first double bond relative to the methyl-end (*n*-).

[c]Values represent the percentage of total fatty acids and are expressed as least squares means across all sampling times; means with a superscript that do not have a common superscript letter (f, g, and h) differ ($P < .05$). Only fatty acids that accounted for ≥ 1% of the total are presented. The following fatty acids had a significant diet × time interaction: 18:0, 20:4*n*-6, and 20:5*n*-3. For these latter two fatty acids this interaction is illustrated in Figures 1 and 2. The time-dependent changes in 18:0 levels were small (2 to 4%) within treatment groups and are not shown.

[d]SAT = sum total area percentage of 14:0, 16:0, 18:0; MONO = sum total area percentage of 16:1*n*-7, 18:1*n*-7, 18:1*n*-9, and 20:1*n*-9; PUFA = sum total area percentage of 18:2*n*-6, 18:3*n*-6, 18:3*n*-3, 20:2*n*-6, 20:3*n*-6, 20:4*n*-6, 20:5*n*-3, 22:4*n*-6, 22:5*n*-6, 22:5*n*-3, and 22:6*n*-3.

[e]Total *n*-6 = sum total area percentage of 18:2*n*-6, 18:3*n*-6, 20:2*n*-6, 20:3*n*-6, 20:4*n*-6, 22:4*n*-6, and 22:5*n*-6; total *n*-3 = sum total area percentage of 18:3*n*-3, 20:5*n*-3, 22:5*n*-3, and 22:6*n*-3.

(8.7 vs 12.6%) for the LA group of pig and sows, respectively). However, as in the sow, pigs suckling sows fed fish oil lowered this *n*-6 PUFA to approximately one half of its original level.

Implications

Feeding fish oil to the sow leads to the enrichment of the suckling pig with omega-3 polyunsaturated fatty acids. Some of these fatty acids were transferred in utero, but the bulk reach the suckling pig through the sow's milk. The amount of omega-3 polyunsaturated fatty acids passing through the milk and into the blood of the nursing pigs may be sufficient to alter eicosanoid production. This, in turn, may alter immune function and incidence of inflammatory disease in these pigs.

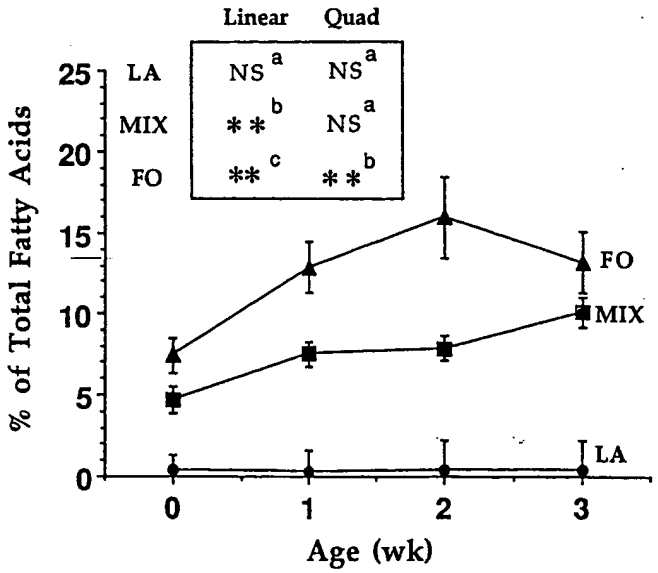

Figure 3. Serum eicosapentaenoic acid (EPA) levels over time in pigs suckling sows fed two levels of fish oil. Conditions and data analysis were as described in Figure 2. Values for pigs suckling sows fed fish oil (FO) and the 1:1 mix of lard and fish oil (MIX) were significantly different ($P < .05$) from those for the lard-fed (LA) group at all time points, but MIX and FO were different from each other at wk 1 and 2. The following regression equations best fit the FO and MIX data, respectively: $Y_{(FO)} = -3.125 + 12.425x - 2.075x^2$, $R^2 = .984$; $Y_{(MIX)} = 3.4 + 1.66x$, $R^2 = .934$.

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The Effects of Varying Dietary n-6 to n-3 Fatty Acid Ratios on Platelet Reactivity, Coagulation Screening Assays, and Antithrombin III Activity in Dogs

Thirty beagles were placed on diets containing ratios of n-6 to n-3 fatty acids ranging from 5:1 to 100:1 for 12 weeks to determine the effects of these diets on platelet reactivity, coagulation screening assays, and antithrombin III activity. Although small changes were observed in adenosine diphosphate (ADP)-, collagen-, and arachidonic acid-induced platelet aggregation and ^{14}C-serotonin release, fibrinogen concentrations, and antithrombin III activities during the 12-week study, these changes were not of clinical significance and did not correlate with the varying ratios of n-6 to n-3 fatty acids. **J Am Anim Hosp Assoc 1997;33:235-43.**

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Introduction

Supplementation of diets with n-3 polyunsaturated fatty acids, with the aim of reducing inflammation, thrombosis, or both has been evaluated extensively in humans and experimental animal models.[1-4] The most prevalent n-6 polyunsaturated fatty acid is arachidonic acid, while the most common n-3 polyunsaturated fatty acid is eicosapentaenoic acid.[5] Hydrolysis of arachidonic acid during platelet activation leads to the formation of prostaglandin endoperoxides, PGG_2 and PGH_2, and thromboxane A_2.[6] Thromboxane A_2 is a potent vasoconstrictor and platelet-activating agent. Neutrophils convert liberated arachidonic acid to lipoxygenase products including hydroperoxyeicosatetraenoic acid (HPETE), hydroxyeicosatetraenoic acid (HETE), and leukotrienes. Leukotriene B_4 is a potent inflammatory mediator, having effects on leukocyte chemotaxis, leukocyte activation, and complement receptor expression.[7] In contrast, hydrolysis of eicosapentaenoic acid (EPA) leads to the generation of the prostaglandin endoperoxide PGH_3 and thromboxane A_3, which are considered to have weak or neglible effects on platelet activation.[6] In addition, leukotriene B_4 production by neutrophils is decreased, and leukotriene B_5 production is increased when neutrophil cell membrane incorporation of EPA occurs. Leukotriene B_5 is considerably less potent than leukotriene B_4 in mediating neutrophil activation and chemotaxis.[7]

Studies suggest that fatty acids of the n-3 series compete with fatty acids of the n-6 series for incorporation within cell membranes. Support of the hypothesis that diets high in n-3 polyunsaturated fatty acids reduce the incidence of inflammation/thrombosis in human populations comes from studies evaluating Greenland Eskimos and Japanese residing in fishing villages, whose diets tend to be high in marine fish, an excellent source of n-3 polyunsaturated fatty acids. Greenland Eskimos have exceptionally low mortality due to ischemic heart disease and other cardiovascular disorders, have a low incidence of atherosclerosis and chronic inflammatory diseases, and also have an enhanced bleeding tendency which may be due to impaired

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platelet function.[8-13] In hyperlipidemic states, coagulation factor activity is enhanced, fibrinogen is increased, and there are high plasma levels of thrombin-antithrombin complexes suggestive of a prothrombotic state.[14] By altering dietary fatty acid ratios, changes in coagulation factor activities may occur in addition to changes in platelet reactivity.

Studies in dogs have suggested that n-3 fatty acid supplementation of diets may aid in reduction of inflammatory or allergic skin disease or both.[15,16] These fatty acids are incorporated within cutaneous cell membranes where they compete with arachidonic acid for lipoxygenase enymes after a traumatic or immunological insult. The resulting 15-hydroxyeicosapentaenoic acid (15-HEPE) and leukotriene B_5 products inhibit the production of the proinflammatory product leukotriene B_4. Eicosapentaenoic acid also may compete with arachidonic acid for the cyclooxygenase enzyme resulting in decreased production of the proinflammatory product prostaglandin E_2.[16]

Current, commercially available, over-the-counter canine and feline nutritional products have n-6 to n-3 fatty acid ratios which range from approximately 5:1 to 100:1. The recent phenomenon of high dietary inclusion rates of vegetable oils, such as corn oil and safflower oil, as a source of the essential fatty acid linoleic acid (18:n-6) has resulted in very high n-6 to n-3 fatty acid ratios in selected products. A screening of commercially available products in 1991 revealed that n-6 to n-3 fatty acid ratios ranged from approximately 15:1 to 85:1 (The Iams Company, unpublished data). The n-3 fatty acid adjusted diets, such as Eukanuba brand products, have n-6 to n-3 ratios between 5:1 and 10:1.

The optimum ratio of n-6 to n-3 fatty acids in diets of dogs recently was evaluated in a group of 30 beagles.[17] Experimental diets ranged in n-6 to n-3 ratios from 5:1 to 100:1. Diets containing n-6 to n-3 ratios of 5:1 and 10:1 were found to be optimal based on skin and neutrophil synthesis of leukotriene B_4 and B_5. Effects of these diets on platelets, coagulation, or thrombosis, however, had not been reported. The purpose of this report is to present information on the effects of experimental diets containing various ratios of n-6 to n-3 fatty acids on platelet reactivity and coagulation screening tests in dogs.

Materials and Methods

Animals and Diets

Thirty, one-year-old, purpose-bred beagles were used in the study. Dogs were divided into five groups (A through E), with each group containing six dogs (three males and three females per group). The n-6 to n-3 ratios in the experimental diets were 5:1, 10:1, 25:1, 50:1, and 100:1 for groups A through E, respectively. All dogs were fed a basal diet containing an n-6 to n-3 ratio of 28:1 for eight weeks prior to beginning the 12-week study using the respective experimental diets. The experimental diets were formulated to contain the same crude protein, crude fat, and crude fiber levels, with varying n-6 to n-3 fatty acid ratios between treatments. Fresh chicken, chicken by-product meal, corn, and rice were kept constant across all experimental diets. Menhaden oil (concentrated source of EPA, 20:5n-3, and docosahexaenoic acid, 22:6n-3), full fat flax (concentrated source of α-linolenic acid, 18:3n-3), and safflower oil (concentrated source of linoleic acid, 18:2n-6), were used to produce increasing dietary n-6 to n-3 fatty acid ratios. Actual laboratory analysis of feeds revealed n-6 to n-3 fatty acid ratios of 5.3:1, 10.4:1, 24.1:1, 51.6:1, and 95.8:1, respectively. Varying combinations of fat sources were used for the 5:1 ratio (1.8% menhaden oil, 11.0% chicken fat, 0.4% flax), 10:1 ratio (0.8% menhaden oil, 12.0% chicken fat, and 0.2% flax), 25:1 ratio (13.0% chicken fat), 50:1 ratio (9.7% chicken fat, 3.3% safflower oil), and 100:1 ratio (9.0% safflower oil, 4.0% chicken fat) experimental diets. The basal diet was formulated with fresh chicken and chicken by-product meal as protein sources, corn and rice as carbohydrate sources, and chicken fat as the sole supplemental lipid source (approximately 13.0% supplemental chicken fat added). Dogs did not receive any other supplements or medication throughout the trial. All procedures were approved by the Auburn University Animal Care and Use Committee.

Platelet Isolation

Eighteen milliliters of blood were collected from the jugular vein of each awake, nonsedated dog into two, 12-ml plastic syringes, each containing 1 ml of 3.8% trisodium citrate. Whole blood platelet counts and mean platelet volumes were determined within 30 minutes of blood collection using an automated instrument[a] prior to preparation of platelet-rich plasma (PRP). Platelet-rich plasma was prepared at 21° C by centrifuging blood for three or four two-minute intervals at 600 to 700 x g and pooling the serially collected supernatants. Platelet-poor plasma (PPP) was prepared by centrifuging the remaining blood at 2,200 x g for 15 minutes or by centrifuging an aliquot of PRP for two minutes at 15,000 x g in an Eppendorf centrifuge.[b] Platelet-rich plasma was diluted to 300,000/µl with autologous PPP prior to evaluation of function.

Platelet Aggregation

Platelet aggregation was performed with a dual-channel platelet aggregometer[c] linked to a strip chart recorder[d] as described.[18] Three concentrations of adenosine diphosphate (ADP; 25-, 10-, and 5-µM final)

and three concentrations of collagen (12-, 6-, and 3-µg/ml final) were evaluated at each sampling period. These concentrations of agonists were found to induce maximal, moderate, and minimal platelet aggregation responses in the majority of normal canine platelet samples in a prior study.[19] In addition to collagen and ADP, aggregation responses to 20-µM epinephrine, 1-mM arachidonic acid, and a combination of epinephrine and arachidonic acid were evaluated. For the combination experiments, epinephrine was added to the platelet suspension first, and the platelets were allowed to stir for one minute prior to the addition of arachidonic acid. For all experiments, the maximal change in light transmittance occurring within a three-minute period was determined, using the point of maximal shape change as baseline.

Platelet ^{14}C-Serotonin Release

Platelet release was evaluated concurrently with evaluation of platelet aggregation by use of the technique of Jerushalmy and Zucker,[20] with modifications. Platelet-rich plasma was radiolabeled at 21° C for 30 minutes by adding 2 µl of a ^{14}C-serotonin stock solution[e] (0.008 µCi/µl) per ml of PRP. At the end of 30 minutes, 3-µM imipramine (final concentration) was added to the platelets to prevent serotonin reuptake during experiments. Radiolabeled PRP (450 µl) was evaluated for three minutes in the platelet aggregometer following addition of agonist. At the end of three minutes, 125 µl of cold formaldehyde (633 mM in 54 mM ethylenediaminetetraacetic acid-0.15 M sodium chloride) were added to the cuvette, and the cuvette was immersed in an ice bath. Cooled samples were centrifuged immediately in the Eppendorf centrifuge for one minute at 16,000 x g, and duplicate 50-µl aliquots of supernatant were removed for counting in a liquid scintillation counter.[f] Total platelet ^{14}C-serotonin uptake was determined by evaluating samples of nonactivated whole PRP and PRP supernatant. Saline solution (0.15 M) was used to determine the amount of ^{14}C-serotonin released in response to stirring alone.

Reagents

Collagen[g] was prepared as described.[21,22] The collagen concentration of the stock solution, as determined by hydroxyproline analysis, was 240 µg/ml. Collagen aliquots were stored at -80° C. Adenosine diphosphate[h] was dissolved in 0.15 M sodium chloride (NaCl, 1,000-µM stock), and aliquots were stored at -20° C. ^{14}C-serotonin was diluted to 0.008 µCi/µl in 95% ethanol and stored at -20° C. Arachidonic acid was dissolved in 0.05 M tris-0.15 M NaCl, pH 7.4 (10-mM stock), and aliquots were purged with oxygen-free nitrogen prior to freezing at -80° C. Epinephrine[j] was solubilized in deionized water (1,000-µM stock) and frozen in 2-ml aliquots at -20° C. All reagents were thawed and kept on ice immediately prior to use.

Coagulation Screening Tests

The prothrombin time (PT),[k] activated partial thromboplastin time (APTT),[l] and thrombin time[m] were performed on citrated plasma using a fibrometer[n] to detect fibrin formation. A standard curve for fibrinogen values was generated by plotting dilutions of a fibrinogen calibration reference solution versus thrombin time values on log-log paper. This standard curve was used to derive fibrinogen values from the thrombin time values obtained from experimental samples. Samples, including controls, were evaluated in duplicate.

Antithrombin III

Antithrombin III activity in citrated plasma samples was determined with an automated chemical analyzer[o] using a chromogenic substrate.[p] Normal, pooled canine plasma was used to generate a six-point standard curve.

Statistical Analysis

Data obtained at six and 12 weeks was compared to data obtained at baseline within groups and between groups using a repeated measures analysis of variance (ANOVA).[q]

Results

Platelet Number and Volume

Platelet number and volume did not differ significantly between groups at baseline. Platelet number decreased slightly (p less than 0.05) in groups C and E after 12 weeks. Thrombocytopenia or thrombocytosis, however, was not documented in any sample taken at any time during the study. Mean platelet volume decreased in groups C and D after 12 weeks (p less than 0.05); however, the decrease was not below the reference range for the laboratory [Table 1].

Platelet Aggregation and ^{14}C-Serotonin Release

Platelet aggregation responses to all agonists at all concentrations were not significantly different between groups at baseline. Twenty-five micromolar ADP-induced platelet aggregation responses were diminished in groups A and D after six weeks (p less than 0.05) [Table 2]. Group D continued to have diminished platelet aggregation responses at 12 weeks (p less than 0.05). Ten micromolar ADP-induced platelet aggregation responses were diminished after six weeks in groups A, C, D, and E (p less than 0.05). Groups A, C, and D continued to have diminished aggregation responses after 12 weeks (p less than

Table 1

Platelet Numbers and Mean Platelet Volumes of Dogs Fed Varying Ratios of n-6 to n-3 Fatty Acids*

Platelet Number† (x10³/µl) Group	Baseline	6 weeks	12 weeks
A	341±22‡	340±30	317±25
B	334±28	311±33	321±19
C	353±34	356±44	285±41§
D	371±19	339±20	352±28
E	325±30	314±28	246±28§

Mean Platelet Volume‖ (fl) Group	Baseline	6 weeks	12 weeks
A	6.4±0.2	6.1±0.3	6.0±0.2
B	6.2±0.2	6.1±0.2	5.8±0.1
C	6.4±0.4	6.3±0.3	5.9±0.2§
D	6.4±0.1	6.3±0.1	6.0±0.1§
E	6.4±0.3	6.5±0.3	6.1±0.4

* n-6 to n-3 ratios by group: Group A=5:1; Group B=10:1; Group C=25:1; Group D=50:1; Group E=100:1

† Reference range, 200 to 500x10³/µl

‡ Mean±standard error of the mean (SEM)

§ Significantly different (p less than 0.05) from within group baseline

‖ Reference range, 5.6 to 8.8 fl

0.05). Five micromolar ADP-induced platelet aggregation responses were decreased in groups A and E after six weeks and in groups C and D after 12 weeks (p less than 0.05).

Twelve micrograms/milliliter collagen-induced platelet aggregation responses were decreased in groups A, D, and E after six weeks and in group D after 12 weeks (p less than 0.05) [Table 3]. Six micrograms/milliliter collagen-induced platelet aggregation responses were decreased in groups A and E after six weeks (p less than 0.05) and in none of the groups after 12 weeks. Three micrograms/milliliter collagen-induced platelet aggregation responses were decreased in group B after six and 12 weeks (p less than 0.05). Collagen-induced ^{14}C-serotonin release responses paralleled aggregation responses [Table 3].

Arachidonic acid (1.0 mM) induced a weak, reversible aggregation response at baseline [Table 4]. Arachidonic acid-induced platelet aggregation responses were diminished further in groups B and E after six weeks and in group E after 12 weeks (p less than 0.05). Changes were not observed in arachidonic acid-induced platelet serotonin release. Epinephrine (20 µM) greatly enhanced arachidonic acid-induced platelet aggregation and ^{14}C-serotonin release in all groups throughout the study [Table 4]. A mild decrease in potentiation of the aggregation response was seen after 12 weeks in group E (p less than 0.05). This was not accompanied by a decrease in ^{14}C-serotonin release. Epinephrine (20 µM) alone failed to induce platelet shape change, aggregation, or release at any point in the study (data not shown).

Coagulation/Antithrombotic Parameters

Prothrombin times and APTTs remained within the normal ranges in all groups throughout the study [Table 5]. Fibrinogen decreased slightly in group C after six and 12 weeks; however, fibrinogen concentrations never decreased below the reference range [Table 5]. Antithrombin III activity decreased in groups A, B, C, and D after 12 weeks; activities, however, did not decrease below the normal range [Table 5].

Discussion

Diets providing increased concentrations of EPA (n-3 fatty acids) may cause changes in platelet membrane fatty acid composition, resulting in changes in membrane fluidity,[1] platelet procoagulant activity,[23] and the ability of platelets to synthesize prostaglandins, thromboxanes, and leukotrienes.[24] These changes may be reflected in *in vitro* tests of platelet aggregability. Although several prior studies in humans have demonstrated that populations ingesting diets high in n-3 fatty acids tend to have decreased platelet aggrega-

Table 2

Adenosine Diphosphate (ADP)-Induced Platelet Aggregation Responses of Dogs Fed Varying Ratios of n-6 to n-3 Fatty Acids*

25 μM ADP-Induced Platelet Aggregation (%LT†)			
Group	Baseline	6 weeks	12 weeks
A	46±7‡	27±8§	34±8
B	52±7	43±8	47±7
C	45±8	44±6	41±9
D	55±9	44±9§	38±7§
E	48±10	34±7	40±10

10 μM ADP-Induced Platelet Aggregation (%LT)			
Group	Baseline	6 weeks	12 weeks
A	32±7	19±6§	22±5§
B	38±6	32±7	31±6
C	36±9	28±7§	30±8§
D	40±8	29±8§	28±8§
E	35±9	23±6§	28±7

5 μM ADP-Induced Platelet Aggregation (%LT)			
Group	Baseline	6 weeks	12 weeks
A	22±5	11±3§	14±4
B	26±5	23±5	23±4
C	25±6	18±3	16±3§
D	27±6	21±6	17±4§
E	24±6	13±2§	16±4

* n-6 to n-3 ratios by group: Group A=5:1; Group B=10:1; Group C=25:1; Group D=50:1; Group E=100:1

† %LT=percent light transmittance

‡ Mean±standard error of the mean (SEM)

§ Significantly different (p less than 0.05) from within group baseline

tion responses to ADP, collagen, and arachidonic acid,[25–27] the changes observed were mild and at times were reversible even with continued ingestion of the diet.[28] In some instances, no change in platelet reactivity was observed;[8] in others, increased platelet aggregability was observed.[29] Variables such as duration of intake of the diet, the ratio of n-6 to n-3 fatty acids, the formulation of the diet (i.e., the source of n-3 fatty acids), and laboratory techniques all may have contributed to the different effects seen. Even in studies demonstrating a reduction in platelet aggregability, however, the effects were mild and not likely to have had clinical significance. Other changes that have been observed with fish oil-supplemented diets in humans include reduced plasma levels of cholesterol, triglyceride, β-thromboglobulin, and antithrombin III as well as a decrease in systemic blood pressure, whole blood viscosity, and platelet retention in glass-bead columns.[13]

In studies with rats and rabbits, the feeding of menhaden fish oil containing 14% to 17% EPA resulted in a decrease in platelet reactivity and a decrease in thrombotic occlusion of extracorporeal shunts.[13] Supplementation of feline diets with menhaden oil caused a reduction in the area of cerebral infarct after middle cerebral artery occlusion.[13] Dogs supplemented with menhaden oil had reduced cardiac infarct size and a reduced number of ectopic beats after electrically induced coronary artery thrombosis.[13] Changes in *ex vivo* platelet reactivity were not observed in the latter study. Dietary fish oil also ameliorated acute renal failure in an experimental uninephric dog model[30] and enhanced the rate of recombinant tissue-type plasminogen activator-induced coronary throm-

Table 3

Collagen-Induced Platelet Aggregation and ^{14}C-Serotonin Release Responses of Dogs Fed Varying Ratios of n-6 to n-3 Fatty Acids*

12 µg/ml Collagen-Induced Platelet Aggregation (%LT†) and ^{14}C-Serotonin Release (%R‡)

	%LT			%R		
Group	Baseline	6 weeks	12 weeks	Baseline	6 weeks	12 weeks
A	58±1§	29±12‖	40±9	55±8	33±9‖	44±6
B	63±4	46±9	51±10	62±4	44±7	50±8
C	58±5	58±5	52±5	58±3	56±3	49±4
D	56±9	43±11‖	36±9‖	56±7	42±9‖	44±8‖
E	57±8	31±7‖	46±8	57±5	32±2‖	50±5

6 µg/ml Collagen-Induced Platelet Aggregation (%LT) and ^{14}C-Serotonin Release (%R)

	%LT			%R		
Group	Baseline	6 weeks	12 weeks	Baseline	6 weeks	12 weeks
A	44±9	18±9‖	27±8	38±5	18±7‖	26±6
B	46±7	24±9	33±12	40±5	24±7	28±7
C	41±6	43±8	31±8	34±4	33±5	28±5
D	45±12	35±12	31±9	36±8	28±8	30±6
E	40±8	7±3‖	33±8	34±4	11±3‖	28±3

3 µg/ml Collagen-Induced Platelet Aggregation (%LT) and ^{14}C-Serotonin Release (%R)

	%LT			%R		
Group	Baseline	6 weeks	12 weeks	Baseline	6 weeks	12 weeks
A	16±5	6±4	5±3	15±3	7±4	8±2
B	24±8	5±3‖	11±6‖	18±4	7±2‖	11±3‖
C	10±5	25±7	5±2	10±3	20±4	8±2
D	15±7	3±2	3±1	14±5	5±1	7±1
E	12±5	2±2	8±4	12±3	5±1	9±2

* n-6 to n-3 ratios by group: Group A=5:1; Group B=10:1; Group C=25:1; Group D=50:1; Group E=100:1

† %LT=percent light transmittance

‡ %R=percent release

§ Mean±standard error of the mean (SEM)

‖ Significantly different (p less than 0.05) from within group baseline

bolysis in a canine model.[31] The protective effects of fish oil were considered to be a result of changes in prostanoid metabolism, including a reduction in generation of thromboxane A_2. The inhibition of thromboxane A_2 synthesis is not linear to thromboxane-dependent platelet activation, with 90% inhibition of synthesis required before an effect on platelet reactivity can be observed.[32] It is conceivable, therefore, that fish oil-supplemented diets may inhibit thromboxane A_2 generation without having a demonstrable effect on *ex vivo* platelet reactivity.

In this study, slight decreases in arachidonic acid-, ADP-, and collagen-induced platelet aggregation and ^{14}C-serotonin release were observed in dogs fed diets having variable ratios of n-6 to n-3 fatty acids. Changes were not clinically significant and were not consistent with respect to fatty acid ratio. In several instances, changes observed at six weeks were not observed after 12 weeks, implying that the changes were reversible. Platelet number and mean platelet volume decreased slightly in groups fed diets with n-6 to n-3 ratios of 25:1 or greater; however, values always remained within the normal reference ranges

Table 4

Arachidonic Acid- and Epinephrine+Arachidonic Acid-Induced Platelet Aggregation and ^{14}C-Serotonin Release Responses of Dogs Fed Varying Ratios of n-6 to n-3 Fatty Acids*

1 mM Arachidonic Acid-Induced Platelet Aggregation (%LT†) and ^{14}C-Serotonin Release (%R‡)

	%LT			%R		
Group	Baseline	6 weeks	12 weeks	Baseline	6 weeks	12 weeks
A	4±1§	1±1	2±1	2±1	2±1	2±1
B	5±1	1±1‖	4±1	3±1	2±1	3±1
C	4±1	2±2	3±1	2±1	2±1	1±1
D	7±3	4±1	2±1	3±1	3±1	2±1
E	6±1	2±1‖	2±1‖	3±1	4±1	2±1

20 μM Epinephrine+1 mM Arachidonic Acid-Induced Platelet Aggregation (%LT) and ^{14}C-Serotonin Release (%R)

	%LT			%R		
Group	Baseline	6 weeks	12 weeks	Baseline	6 weeks	12 weeks
A	86±2	88±3	82±4	35±4	32±4	25±6
B	88±3	90±3	80±4	30±4	30±4	32±4
C	82±4	88±3	82±1	28±5	30±4	29±6
D	88±4	86±5	84±4	34±7	31±9	27±7
E	86±3	82±2	75±2‖	29±4	29±4	33±5

* n-6 to n-3 ratios by group: Group A=5:1; Group B=10:1; Group C=25:1; Group D=50:1; Group E=100:1

† %LT=percent light transmittance

‡ %R=percent release

§ Mean±standard error of the mean (SEM)

‖ Significantly different (p less than 0.05) from within group baseline

and were not clinically significant. Decreased platelet number has been observed in humans receiving diets high in n-3 fatty acids; however, platelet counts did not decrease below the physiologic range.[26]

In the present study, clinically significant effects of n-3 fatty acids were not seen on coagulation screening tests (PT and APTT), on fibrinogen concentration, or on antithrombin III activity. Changes in fibrinogen and antithrombin III activity have been observed in some studies in humans with fish oil-supplemented diets.[13,33] The lack of effect seen in the authors' study may be related to the dietary ratio of n-6 to n-3 fatty acids, to the concentration and type of n-3 fatty acids used, or both.

Conclusion

The diets used in this study did not have a clinically significant effect on platelet reactivity, coagulation protein screening assays, fibrinogen, or antithrombin III activity in purpose-bred laboratory beagles. The alterations in n-6 to n-3 fatty acid ratios in the diets described in this study may result in long-term, subtle effects on platelet reactivity; however, these effects are not likely to manifest as a clinically significant bleeding tendency. These findings are encouraging and suggest that diets formulated with n-6 to n-3 fatty acid ratios of 5:1 or 10:1, which previously have been shown to alter canine neutrophil production of leukotriene B_4, may be beneficial in reducing inflammatory skin disease with minimal risk of promoting a bleeding tendency.

a Series 810; Baker Instruments Corp., Allentown, PA

b Model 5414; Hamburg, Federal Republic of Germany

c Model 450; Chrono-log Corp., Havertown, PA

d Model 707; Chrono-log Corp., Havertown, PA

e 5-Hydroxytryptamine creatinine sulfate (53 mCi/mMol); Amersham Corp., Arlington Heights, IL

f Model LS 7000; Beckman Instruments, Inc., Irvine, CA

g Type I, Bovine Achilles tendon, Sigma Chemical Co., St. Louis, MO

h Grade I, Sigma Chemical Co., St. Louis, MO

i Nu Chek Prep, Elysian, MN

j Sigma Chemical Co., St. Louis, MO

k Thromboplastin C; Baxter Health Care Corp., Miami, FL

l Actin activated cephaloplastin reagent; Baxter Health Care Corp., Miami, FL

(Continued on next page)

Table 5

Prothrombin Times, Activated Partial Thromboplastin Times, Fibrinogen Concentrations, and Antithrombin III Activities in Dogs Fed Varying Ratios of n-6 to n-3 Fatty Acids*

Prothrombin time† (PT; seconds)

Group	Baseline	6 weeks	12 weeks
A	6±0.1‡	6±0.1	7±0.1
B	7±0.2	7±0.2	7±0.2
C	6±0.1	7±0.1	7±0.1
D	7±0.3	7±0.1	7±0.2
E	7±0.2	7±0.2	7±0.2

Activated partial thromboplastin time† (APTT; seconds)

Group	Baseline	6 weeks	12 weeks
A	10±0.4	9±0.1	9±0.1
B	9±0.2	10±0.4	9±0.1
C	9±0.2	10±0.2	9±0.3
D	9±0.1	10±0.1	10±0.2
E	10±0.4	10±0.1	10±0.2

Fibrinogen† (mg/dl)

Group	Baseline	6 weeks	12 weeks
A	187±32	171±11	158±20
B	151±11	155±10	153±13
C	181±9	136±7§	134±7§
D	188±20	160±8	150±11
E	150±8	129±9	132±15

Antithrombin III activity† (%)

Group	Baseline	6 weeks	12 weeks
A	131±2	133±4	110±2§
B	130±3	127±4	111±3§
C	131±1	130±5	113±2§
D	133±3	136±4	115±3§
E	130±1	135±2	118±6

* n-6 to n-3 ratios by group: Group A=5:1; Group B=10:1; Group C=25:1; Group D=50:1; Group E=100:1

† PT reference range=6.0 to 10.0 sec; APTT reference range=8.0 to 14.0 sec; fibrinogen reference range=100 to 400 mg/dl; antithrombin III reference range=85% to 125%

‡ Mean±standard error of the mean (SEM)

§ Significantly different (p less than 0.05) from within group baseline

[m] Fibrinogen; Sigma Chemical Co., St. Louis, MO

[n] Becton, Dickinson & Co., Rutherford, NJ

[o] Cobas Mira; Roche Diagnostic Systems, Nutley, NJ

[p] S-2238, COATEST antithrombin; Helena Laboratories, Beaumont, TX

[q] ABstat; Anderson-Bell Corp., Parker, CO

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Effects of altering dietary fatty acid composition on prostaglandin synthesis and fertility

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Summary Several studies over the past 20 years have demonstrated that subjects on diets composed of substances with high levels of n-3 polyunsaturated fatty acids (PUFAs) (e.g. fish) have a decreased incidence of heart disease. On this basis, a recent report from the Department of Health has advised UK consumers to decrease the proportion of saturated as opposed to unsaturated fats in their diet and to increase the ratio of n-3 to n-6 PUFAs. This could be achieved by altering the amounts of these constituents in milk and meat. n-3 Fatty acids can most easily be added to animal feed as either fish oil or linseed oil and can be increased in the blood and milk of ruminants following protection to avoid hydrogenation in the rumen. In western countries the ratio of consumption of n-6 to n-3 PUFAs is greater than 10 and current evidence tends to suggest that a ratio nearer 5 would be more desirable and compatible with cardiovascular well being. As fertility in the UK dairy herd is already poor, it is important to establish whether alterations in dietary n-3 and n-6 PUFAs affects herd fertility before widespread changes in animal diets are recommended. Therefore, this review considers the role played by PUFAs and eicosanoids in fertility, with particular reference to the implications for farm livestock production.

The evidence reviewed shows that alteration of the concentration and ratio of n-6 and n-3 PUFAs in feeds can influence prostaglandin synthesis/metabolism in a number of mammalian systems. The changed patterns of prostaglandin synthesis can as a consequence, affect the diverse functions (e.g. hormone secretion) that are normally mediated via prostaglandins. Similarly, changes in prostaglandin synthesis effected through manipulation of PUFAs has a major bearing on fertility (as PGs affect many reproductive parameters, e.g. ovulation). Several studies in cattle and other mammals, show that feeding or infusing different types of fat with varying PUFA content to females can alter: the number and size of ovarian follicles, the ovulation rate, progesterone production by the corpus luteum, the timing of luteolysis and gestational length. In the male most recent work has focussed on sperm production and experiments in fowl have demonstrated clear effects of dietary PUFAs on both the sperm membrane phospholipid composition and on fertilizing ability. © 1999 Harcourt Publishers Ltd.

BACKGROUND

Numerous studies over the past few years have reported that consumption of a diet high in n-3 polyunsaturated fatty acids (PUFAs) is associated with a decreased incidence of cardiovascular disorders.[1–8] Based on this extensive body of evidence, the UK population has been advised to change their diet such that foods rich in n-3 PUFAs forms a larger component of the diet than at present.[9] In other words, to lower the overall ratio of n-6 PUFA to n-3 PUFA from greater than 10 at present to around 5, which is commensurate with cardiovascular well being.[4] This change could be achieved by modifying the amount of n-3 PUFA contained in the main foods (meat and milk) which comprise the current UK diet. Modifying the n-3 PUFA content of milk and meat could be realised through feeding domestic ruminants (e.g. cattle, sheep) diets rich in n-3 PUFAs. However it is important that any recommendations for change in herd diet does not compromise fertility, as the fertility of the UK dairy herd is already poor and declining at a rate of 1% every 3 years. This review therefore considers the role of

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dietary PUFAs and their related eicosanoid products in regulating fertility with particular emphasis on farm livestock production.

INTRODUCTION

Fatty acids

Fatty acids occur mainly as esters in natural fats and oils. However, they also exist in non-esterified form as free fatty acids (a transport form found in plasma). Fatty acids that occur in natural fats are usually straight chain derivatives and contain an even number of carbon atoms. The chain may be saturated (containing no double bonds) or unsaturated (containing one or more double bonds). Unsaturated fatty acids may be further subdivided as monounsaturated (one double bond), polyunsaturated (PUFAs, two or more double bonds) and eicosanoids.

Animal tissues can synthesize the oleic acid (18:1n-9) family of unsaturated fatty acids (Fig. 1). However linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3), the nutritionally essential fatty acids (EFAs), cannot be synthesized endogenously as the required desaturases are absent.[5,10] They therefore have to be provided in the diet. Mammals were first shown to have an absolute requirement for EFAs in 1929.[11,12] Feeding animals a fat-free diet induced a state of EFA deficiency and a variety of pathophysiologic effects were noted: dermatitis, reproductive inefficiency and papillary necrosis. This deficiency state is characterized by a decrease in n-6 and n-3 PUFAs and by an accumulation of n-9 fatty acids, notably 20:3 (termed Mead acid) which is not a constituent of normal tissues (Fig. 1). The ratio of Mead acid to arachidonic acid has been used to define the deficiency state, with a ratio of greater than 0.4 being considered to be consistent with fatty acid deficiency.[13] The body has different requirements for n-6 and n-3 PUFAs, as they are involved in several, yet varied essential functions. For instance, docosahexaenoic acid (DHA 22:6n-3) derived from linolenic acid (18:3n-3) is essential for brain development and visual function,[14] whereas arachidonic acid (AA 20:4n-6) derived by elongation and desaturation from linoleic acid (18:2n-6), is the precursor of eicosanoids and is essential for neonatal growth.[15]

Linoleic acid is the precursor for AA, the unsaturated fatty acid from which the biologically active eicosanoids are derived.[16] Consuming diets deficient in linoleic acid lead to a decrease in tissue and blood arachidonic acid,[1] which results in poorer growth. It is to be expected that a decrease in the precursor (linoleic acid) would lead to a decrease in the product formed (AA). However, abundance of the precursor (linoleic acid) does not necessarily reflect the amount of product (AA) in certain species, as linoleic acid is not efficiently desaturated and elongated to AA in humans.[17,18] Therefore, while linoleic acid (which is abundant in nearly all commonly available vegetable oils, e.g. corn, sunflower, safflower and rape seed oils)[8] is the precursor for AA, the majority of the AA needed by carnivores is obtained from animal products.[18] In herbivores such as cows, the AA needed is provided equally by: (1) dietary intake of linoleic acid and (2) de novo synthesis from acetate and β-hydroxybutyrate (via fatty acid synthetases).[19,20] PUFA deprivation causes a general decrease in levels of AA, although the effects on the phospholipid composition of different tissues can be quite diverse. For example, it was found that AA concentrations in liver lipids were depleted, those in renal cortical lipids were unchanged, while the AA content of heart lipids actually increased.[21] Moreover, three physiological functions (dermal integrity, renal function and parturition) appeared to have a greater dependence on n-6 PUFAs, since they were better maintained with n-6 fatty acids when compared to their maintenance with n-3 fatty acids.[12,22]

Like the AA of the n-6 PUFAs, the long chain (n-3) PUFAs, DHA (22:6n-3) and eicosapentaenoic acid (EPA 20:5n-3) are also essential for many bodily functions. They can be delivered directly from the diet or produced within the body from the precursor α-linolenic acid.[8] α-Linolenic acid is present in all green leaf vegetables as a component of chloroplast lipids, although these lipids constitute only a small fraction of green leaf biomass. Linseed oil is one of the few vegetable oils that contain high levels of α-linolenic acid but it also contains significant quantities of linoleic acid.[8] α-Linolenic acid is present in grass, although concentrations are reduced during silage making. Fish oils are also high in α-linolenic acid and currently offer the most readily available dietary source of DHA and EPA.[23]

An interesting difference between plants and animals with regard to synthesis of PUFAs concerns the desaturation of oleic acid (n-9) to linoleic acid (n-6) and α-linolenic acid (n-3). In plants, oleic acid can be converted to linoleic and linolenic acids via the appropriate desaturases, whilst in animals the absence of these desaturases prevents linoleic and linolenic acid from being formed. In all animals including humans, desaturation takes place in the direction of the carboxyl group. This means that in animals, interconversion of the families of PUFAs does not take place.[5,17]

The fatty acid composition of blood, tissue and milk in non-ruminants generally reflects the fatty acid content of the diet. In contrast, while the diet of ruminants contains predominantly unsaturated fatty acids, the fat content of blood, tissues and milk is highly saturated. This difference can be explained by the extensive biohydrogenation of unsaturated fatty acids, which occurs in the rumen, through the activity of rumen microorganism's.[24]

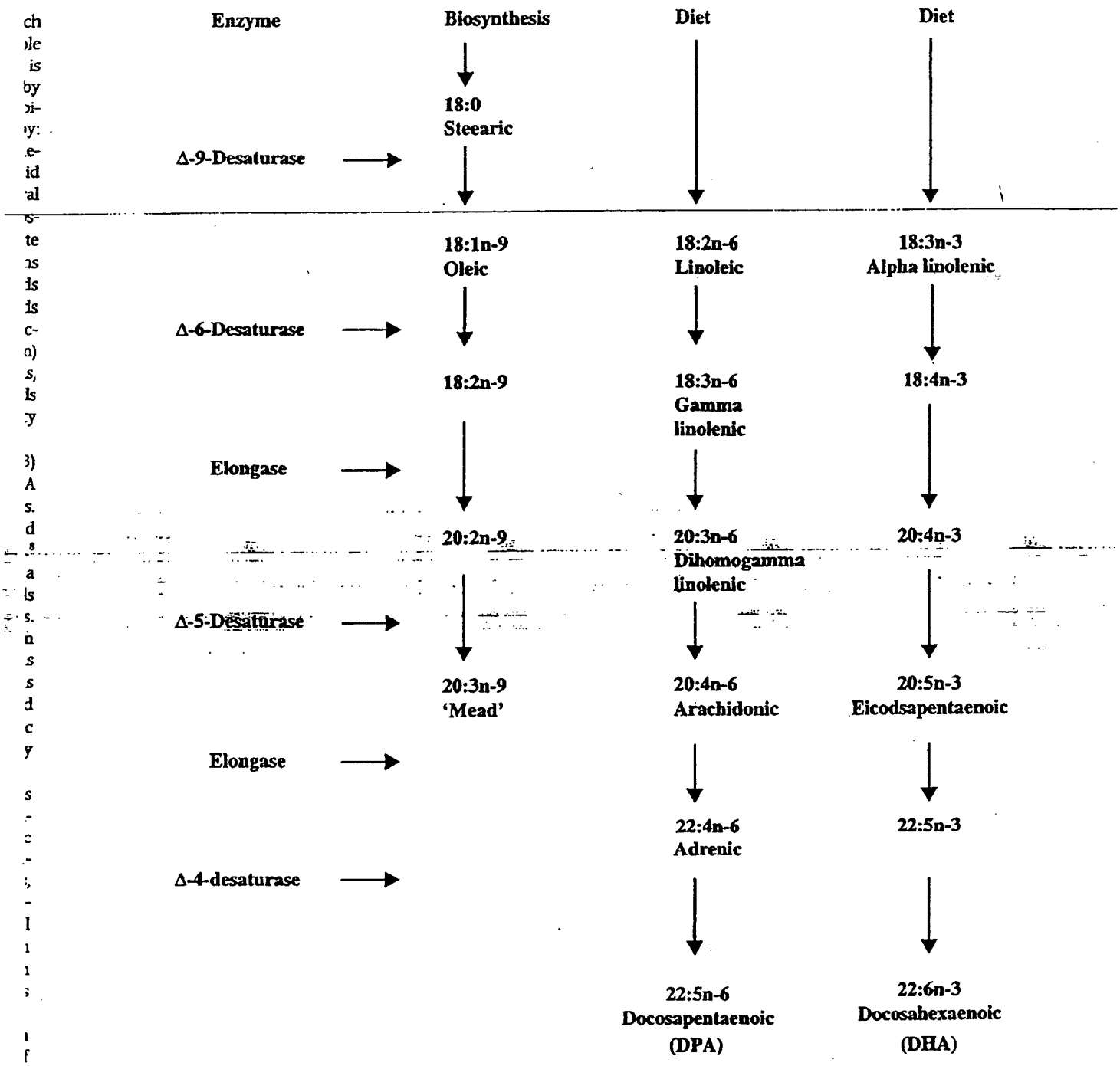

Fig. 1 Metabolic transformations of the three major unsaturated fatty acid families by desaturation and elongation.

Thus, in order to increase the supply of specific unsaturated fatty acids to the blood and tissues of ruminant animals, it is necessary to locate a dietary source rich in the chosen fatty acids and to protect the fatty acid from hydrogenation in the rumen. A number of techniques have been successfully developed to protect fats and oils which involve either chemical (e.g. formaldehyde or calcium salts) or physical (e.g. heat) treatment processes.[25,26]

Eicosanoids

The eicosanoids are derived from eicosanoic (C_{20}) fatty acids and comprise the prostaglandins, thromboxanes, leukotrienes and lipoxins.[13,27] As this review focuses on prostaglandins, they will be the only eicosanoids considered hereafter. Prostaglandins have been implicated in many reproductive functions and are reviewed below. They are also important for a variety of other physiological activities including controlling platelet aggregation and vascular homeostatis,[28] kidney function[27,29] inflammatory and immune responses,[30,32] hormone secretion (e.g. progesterone,[33] insulin)[34] and cell signalling.[27,35,36] In addition, eicosanoids are involved in the aetiology of several disease processes including hypertension[37] and tumour promotion.[38]

The most biologically active prostaglandins of the 2 series (dienoic prostaglandins) are derived from AA that has as its precursor linoleic acid.[5] In most cells AA is present in various cellular phospholipids in an esterified form and the generation of free AA is a rate-limiting step in eicosanoid synthesis[39] (Fig. 2). AA can be liberated from phospholipids directly via the action of an acyl hydrolase, phospholipase A_2(PLA_2)[40] or indirectly via the coordinated actions of phospholipase C (PLC) and diaclyglyceride lipase. The AA that is released via either of these two mechanisms is either immediately re-esterified or metabolized to: (1) prostaglandins and thromboxanes by prostaglandin synthetase (cyclooxygenase); (2) leukotrienes

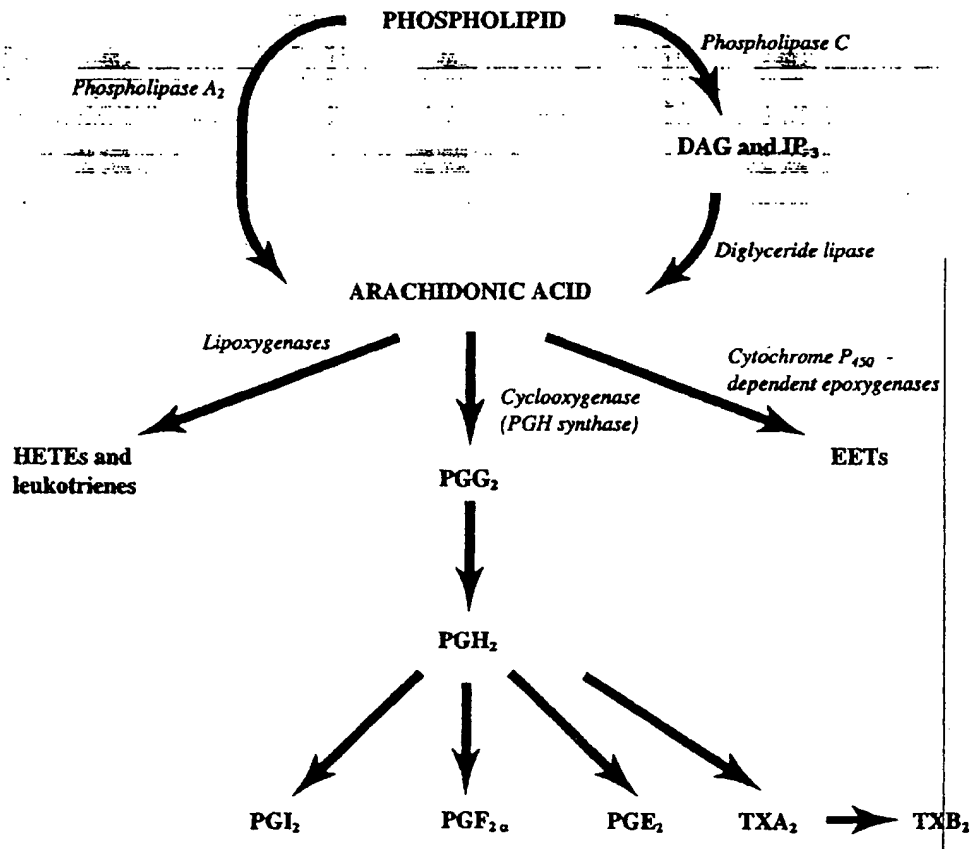

Fig. 2 Pathways of prostaglandin metabolism.

and hydroxy eicosatetraenoic acids (HETEs) by lipoxygenases; or (3) epoxyeicosatrienoic acids (EETs) by cytochrome P450-dependent epoxygenases.[13,27]

Trienoic prostaglandins (3 series prostaglandins) can be formed from α-linolenic acid (18:3n-3) which gives rise to PGE_3 and $PGF_{3\alpha}$. In contrast, the monoenoic prostaglandins (1 series prostaglandins) are derived from dihomogamma linolenic acid (20:3n-6) which gives rise to PGG_1, PGH_1, PGE_1 and $PGF_{1\alpha}$. In general, the 1 and 3 series prostaglandins are considered to be less biologically active than those of the 2 series.[6,19,41] However, as with all generalizations this too has exceptions. For an example, EPA-derived thromboxane A_3 TA_3 (as opposed to AA-derived TA_2)[42] is a weak aggregator of platelets,[43] whereas EPA-derived PGI_3 is as potent[41] as AA-derived PGI_2 as an antiaggregator.[44] Moreover, predicting the changes in the pattern of prostaglandin synthesis following dietary modulation of PUFAs is also difficult. For instance, when animals were fed diets rich in gamma linolenic acid (18:3n-6) supplemented with either EPA or DHA (both n-3), monoenoic prostaglandins were found to be increased in all tissues irrespective of the n-3 PUFA used to supplement the diet.[45] Since these animals lacked delta-5-desaturase (Fig. 1), this was a predictable outcome. In contrast, this dietary regime was also associated with changes in tissue phospholipid levels of PUFAs: increases in linoleic (18:2n-6), gamma linolenic (18:3n-6) and dihomogamma linolenic (20:3n-6) acids and a decrease in AA. The changes are not as expected and reinforce the need for experimental verification through monitoring of PUFAs and prostaglandin generation in the tissue(s) of interest in animals exposed to different PUFA diets. Nevertheless, studies such as these clearly demonstrate the potential for modulating the generation of prostaglandins of altered potency by manipulating the PUFA composition of the diet.

EFFECTS OF DIETARY MANIPULATIONS OF ESSENTIAL FATTY ACIDS ON EICOSANOID SYNTHESIS

As eicosanoids have been implicated in numerous normal bodily functions and disease processes, much effort has been devoted to manipulating the dietary intake of PUFAs in a variety of species with a view to modulating synthesis of eicosanoids (review,[46] human,[5] cow,[47], pig,[48] rat,[49] salmon[50]). Three general experimental approaches have been utilized: (1) deprivation of PUFAs in the diet[29] (2) altering the ratio of n-3 to n-6 PUFAs in the diet;[45,47,49,51–57] and (3) altering the dietary intake of the eicosanoid precursor, AA itself.[18,58]

Feeding subjects diets rich in AA increased the plasma phospholipid levels of AA and the urinary excretion of the stable metabolites of prostacyclin (PGI_2) and TA_2.[59] Subjects fed diets rich in AA (n-6) and either DHA or EPA (both n-3), excreted greater quantities of PGI_2, but not TA_2 in urine.[59] In contrast, other studies involving murine macrophages have demonstrated that increased ingestion of n-3 PUFAs in the diet is associated with a decrease in generation of active prostanoids, i.e. prostaglandins of the 2-series.[60] This may be because the n-3 PUFAs replace AA in tissue phospholipids, which when released compete for prostaglandin synthase, and thereby competitively attenuate the rate of formation of 2-series prostaglandins (which are derived from n-6 PUFAs).[19,61] The direct inhibition of prostaglandin synthase activity by either high levels of n-3[62] or n-6 PUFAs[47] may also contribute to the decrease in overall prostaglandin generation. However, it has to be emphasized that caution should be exercised in extrapolating from animal studies into humans and vice versa as PUFAs (e.g. EPA) added in vitro or provided as a dietary supplement affects prostaglandin production differentially. For instance, human endothelial cells in vitro do not transform exogenous EPA into PGI_3,[63] whereas EPA provided as a dietary supplement increases excretion of PGI_3 in humans,[64] but not in rats.[65]

It is, therefore, clear that dietary manipulations of PUFAs can have major effects on eicosanoid production, although these are hard to predict. The relevance of this kind of experimental approach to probing the role of PUFAs in various processes associated with reproduction is considered next.

EFFECTS OF ALTERING DIETARY FATTY ACIDS ON FEMALE REPRODUCTION

Follicular development

The commencement of ovarian cyclicity heralded by the onset of puberty involves the recruitment, development and ovulation of follicles. Although several follicles are recruited at the start of an ovarian cycle, the number of follicles which go on to ovulate is characteristic for each species and ranges from one (e.g. man, cow) to several hundred (e.g. viscacha: *Lagostomus maximus*).[66] A number of studies in cattle have demonstrated that increasing the fat content of the diet increases both the number and size of follicles present in the ovary and in addition shortens the interval to the first ovulation post-partum.[67–73] The control diets employed indicated that it was the fatty acids themselves, rather than the additional energy they provided, which led to stimulation of the ovary. Two mechanisms of action have been suggested. The first is via increased blood cholesterol (both total and high density lipoprotein-cholesterol.[74] As cholesterol is the precursor of all steroids, increased substrate availability may increase follicular steroid synthesis. The following findings support this contention: (1) androstenedione lev-

els were found to be increased in follicular fluid of cows fed a high lipid diet and (2) granulosa cells obtained from follicles of cows fed a high-lipid diet increased progesterone output in vitro.[74] Oestradiol-17β produced by the coordinate actions of the steroidogenic enzymes in theca and granulosa cells, induces granulosa cell proliferation. This in turn would ultimately result in an increase in follicular size.

A second alternative, yet complementary, explanation could be that increased dietary fats led to an increase in AA in phospholipids of ovarian follicular granulosa cells. When released from phospholipids in response to gonadotrophin stimulation,[75] the AA could either have a direct effect on granulosa cells steroidogenesis[76,77] or be metabolized via the cyclooxygenase pathway to yield prostaglandins. These in turn may exert a stimulatory effect on granulosa cell steroidogenesis. The latter suggestion is supported by the observations that gonadotrophins stimulate prostaglandin production in ovarian follicular cells[78] and prostaglandins (PGE_2) in turn, are known to stimulate ovarian steroidogenesis.[79]

Ovulation

Follicular development culminates in the release of oocyte(s) at ovulation. In addition to effects on ovarian follicular steroid synthesis, AA and its metabolites have been implicated in ovulation in many mammalian species (e.g. rabbit,[80] pig,[81] rat,[82] rhesus monkey).[83] Follicular rupture is prevented by cyclooxygenase inhibitors.[83] This inhibitory effect can be overcome by administration of $PGF_{2\alpha}$.[83,84] Altering the dietary intake of n-3 and n-6 PUFAs led to considerable changes in ovulation rates in rats. Animals on a high n-3 diet had increased ovulations in comparison with rats on a control diet, whereas a diet high in n-6 PUFAs caused a decrease in the number of ova released.[49] Both diets caused an increase in PGE production, although the assay used did not distinguish between PGE_2 (derived from n-6 PUFAs, i.e. AA) and PGE_3 (derived from n-3 PUFAs). As high PGE levels are inhibitory, the authors suggest that the increased ovulation rate associated with the n-3 diet may have been due to a greater production of the less biologically active PGE_3 at the expense of the normal PGE_2. In contrast, the decrease in ovulation brought about by a high n-6 diet could be caused by increased production of PGE_2. While this is an attractive hypothesis confirmation will only be provided if: (1) it is clearly demonstrated that a high n-3 diet leads to a change in the ratio of PGE_3: PGE_2 and (2) PGE_2-induced decreases in ovulation can be blocked by PGE_2 receptor antagonists.

The cessation of ovulation associated with menopause is linked to an increase (~15%) in serum cholesterol.[85] The rise in serum cholesterol appears to be related to the dietary intake of PUFAs, as the elevation in plasma cholesterol in women on a high PUFA diet was less than that in women on a lower PUFA intake.[85] This finding offers the intriguing possibility of the existence of an inverse relationship between PUFA intake and plasma cholesterol levels. Unfortunately, this study did not define the type of PUFA (n-3 or n-6) whose intake was altered. Nevertheless, it does suggest that PUFAs affect ovulation, either by modulating levels of (1) prostaglandins and/or (2) serum cholesterol. However, it is unlikely that changes in serum cholesterol influence ovulation directly. Instead, it may be that changes in serum cholesterol reflects alterations in intakes of particular PUFAs as increased consumption of n-3 PUFA has been shown to cause an increase in cholesterol levels in hamsters.[86] The converse may also hold true, i.e. that lower serum cholesterol is associated with a low intake of n-3 PUFA which in turn could affect the ovulatory processes through modulating the generation of 2- and 3-series prostaglandins.

Corpus luteum function

Follicular rupture leads to the formation of the corpus luteum. Tha fat composition of the diet has been suggested to influence luteal function in three different ways: by a direct action on progesterone production; via alteration of the production of eicosanoids within luteal tissue and/or by interaction with the system controlling luteolysis and the maternal recognition of pregnancy.

Lipid infusion to either cattle or sheep during the luteal phase increases serum concentrations of progesterone.[87–90] As discussed previously, this may have been due to raised cholesterol levels providing an increase in available precursor for progesterone biosynthesis. Another possibility is that clearance rates of progesterone from plasma may be reduced.[89] Both soybean oil and olive oil increased circulating progesterone concentrations, although soybean oil was more effective.[90] This elevation in progesterone could have a beneficial effect on fertility, as sub-optimal progesterone concentrations are associated with high return rates in cows.

The corpus luteum also has the capability of producing a variety of eicosanoids, including $PGF_{2\alpha}$, PGE_2 and PGI_2 (prostacyclin) in addition to products of the lipoxygenase pathway, particularly 5-HETE.[91] $PGF_{2\alpha}$ has an inhibitory effect on progesterone production. This was first demonstrated for the sheep corpus luteum[92] and subsequent studies have extended these observations to most mammalian species.[93–97] In contrast, PGE_2 and PGI_2 are luteotrophic[79,93,98–100] and PGE_2 has been shown to influence its own secretion from bovine corpora lutea.[101] Altering the balance of endogenous prostaglandin production within the corpus luteum may therefore alter both the concentration of progesterone produced and the overall length of the luteal phase.

The increase in luteal $PGF_{2\alpha}$ at the time of luteal regression in rats may be due, in part, to an increase in PLA_2 activity.[102] An increase in PLA_2 activity leads to an increase in AA, and hence its metabolites including $PGF_{2\alpha}$ which are then available to exert an inhibitory action on progesterone production. The finding that the bovine corpus luteum contains very high levels of PUFAs, especially AA, esterified in phospholipids[103] supports this notion. Furthermore the levels of n-6 PUFAs (a source for AA) is highest in the mid- and late-luteal phase of corpora lutea of domestic ruminants (sheep,[104] cow[105]). Luteal cells from cows fed a diet high in n-3 PUFA produce significantly greater quantities of progesterone under basal conditions.[106] This suggests that products of n-3 PUFA oxygenation, namely the trienoic prostaglandins, are capable of stimulating progesterone production by luteal cells. Whilst no direct evidence for this contention exists in luteal tissue, the finding that PGE_3 stimulated testosterone synthesis by testis Leydig cells of goldfish, tends to support this hypothesis.[107]

Infusion of lipids to sheep increased serum concentrations of both the $PGF_{2\alpha}$ metabolite PGFM (13, 14-dihydro-15-keto $PGF_{2\alpha}$) and PGE_2. Olive oil was more effective than soybean oil in this respect and also caused a significant shortening of oestrous cycle length.[90] This difference was perhaps surprising as soybean oil contains more of both linoleic and linolenic acid than olive oil. However, linoleic and linolenic acid can also reduce both AA and prostaglandin synthesis via inhibition of the enzymes PLA_2 and cyclooxygenase.[47,108,109] Excess (n-3) or (n-6) PUFA concentrations may therefore down-regulate dienoic prostaglandin synthesis.

Luteolysis and the maternal recognition of pregnancy

As well as direct effects on the corpus luteum, PUFAs can influence luteal activity via interaction with the uterus. Luteal regression in domestic ruminants and pigs is caused by uterine secretion of $PGF_{2\alpha}$.[94,110,111] In cattle and sheep $PGF_{2\alpha}$ is released from the endometrium in response to oxytocin from the corpus luteum.[112] Oxytocin binding to its uterine receptor leads to the activation of both PI-PLC and PLA_2[113,114] which causes release of AA and its subsequent metabolism via cyclooxygenase to yield $PGF_{2\alpha}$. As in other tissues, availability of AA in the endometrium determines the ability of this tissue to synthesise prostaglandins. Addition of AA to bovine endometrial explants in vitro caused a dramatic increase in prostaglandin output.[115] However, the basal output of 2-series prostaglandins (PGE_2, $PGF_{2\alpha}$ and PGI_2) from endometrial explants in vitro of cows fed a diet high in n-6 PUFAs was decreased compared to controls whereas n-3 PUFA dietary supplementation had no effect on the production of dienoic prostaglandins.[116] This highlights the differences in outcome, in terms of prostaglandin output between PUFA addition in vitro and the dietary supplementation with PUFAs.

Abomasal infusion of cattle with tallow (high in PUFAs but with only 2% linoleic acid) increased plasma PGFM concentrations in response to an oxytocin injection in comparison to treatment with yellow grease containing 20% linoleic acid.[47] Yellow grease in fact impaired the ability of the uterus to secrete $PGF_{2\alpha}$, possibly through inhibition of cyclooxygenase. As higher concentrations of linoleic acid have been found naturally in the endometrium of pregnant versus non-pregnant cows,[117] it has been suggested that this may form part of the normal anti-luteolytic mechanism. During the establishment of pregnancy (maternal recognition of pregnancy) in sheep and cattle, the normal anti-luteolytic mechanism is mediated via the indirect actions of trophoblast interferon, IFNτ, which is thought to act principally by inhibiting the expression of endometrial oxytocin receptors.[118–120] IFNτ could also use alternative mechanisms to inhibit $PGF_{2\alpha}$ synthesis via alterations in lipid metabolism. It is possible that IFNτ redirects AA metabolism down the expoxygenase pathway, thereby decreasing the availability of AA for metabolism via the cycloxygenase pathway.[121] If this were the mechanism used, addition of IFNτ to endometrium either in vivo or in vitro should lead to a decrease in basal $PGF_{2\alpha}$ production. This has indeed been demonstrated.[122,123] Moreover, the possible molecular mechanism(s) utilized by IFNτ in directly inhibiting $PGF_{2\alpha}$ synthesis is provided by the findings that oxytocin-induced expression of cyclooxygenase-2 (the inducible form of the enzyme) and prostaglandin F synthase are inhibited by IFNτ in bovine endometrial cells.[124]

Parturition

The involvement of PUFAs in parturition was first demonstrated in rats the 1930s,[125] whereas rats fed on a diet free of EFAs were found to have a prolonged gestation period (by 1 to 3 days). Prostaglandins are key hormones both in terms of cervical ripening and myometrial contractility, which are essential for mammalian parturition.[126] Therefore, the connection can be made between changes in dietary intake of PUFAs and the ensuing changes in gestational length.

Changes in PUFA intake through altering the pattern of prostaglandin production may influence either the timing or efficiency of the onset of labour. Support for this assertion is provided by numerous studies in animals[125–130] as well as humans.[131–133] In general, animals or humans fed diets high in n-3 PUFAs exhibited an increase in gestational length. This has been attributed to the changed pattern of PG synthesis, which gives rise to an increase in the generation of 3-series prostaglandins. Since 3-series

prostaglandins are less potent than the 2-series prostaglandins (e.g. in terms of inducing contraction)[6,132] normally associated with parturition, the suggestion is that the biological activity of these 3-series prostaglandins is insufficient to induce the vigorous myometrial contractions associated with normal labour. That this may be the case is supported indirectly by the finding that rats fed a diet high in linolenic acid (n-3) were able to reproduce normally providing the pups were delivered by caesarean section.[130] The implication therefore is that n-3 PUFA derived 3-series prostaglandins are capable of substituting for n-6 PUFA derived 2-series prostaglandins in all reproductive processes with the exception of parturition.

Evidence exists to support the idea that normal onset of labour is associated with an increase in n-6 PUFA derived dienoic prostaglandins: (1) plasma levels of linoleic and arachidonic acid (both n-6) are higher than those of linolenic acid, EPA and DHA (all n-3) in women in labour;[134] (2) levels of arachidonic acid increased throughout pregnancy, with highest levels being observed during labour, followed by a rapid decline postpartum;[135] and (3) levels of linoleic acid (the precursor of arachidonic acid – Fig. 1) increased in uterine arteries during pregnancy.[136] From the foregoing it is clear that there exists a large body of evidence to support the idea that normal labour is associated with an increase in n-6 PUFA derived 2-series prostaglandins. It would also appear that an increase in intake of n-3 PUFA during gestation leads to the process of parturition being compromised due to an increase in the less potent 3-series prostaglandins and a concomitant decrease in 2-series prostaglandins. There is compelling evidence to support the latter notion: n-3 PUFAs added in vitro to human decidual cell cultures significantly decreased the production of the 2-series prostaglandins PGE_2 and $PGF_{2\alpha}$[137] and infusion of n-3 PUFAs in vivo into pregnant sheep caused a decrease in both maternal and foetal plasma levels of PGE_2.[128] However direct evidence to support the former is rather surprisingly lacking, i.e. no direct measurements of 3-series prostaglandins have been made following augmentation with dietary n-3 PUFAs. In our view these measurements need to be performed before the hypothesis that n-3 PUFAs prevents the normal onset of labour by giving rise to 3-series prostaglandins in the uterus can be accepted as fact.

Lactation

Parturition is followed by lactation. There are numerous studies showing that it is possible to manipulate the fatty acid composition of milk by feeding protected unsaturated fats and oils.[26,138,139] There is considerable interest in this work in relation to the production of milk with a higher unsaturated to saturated fat ratio to benefit human health. This topic merits a review in its own right and is not considered here.

MALE FERTILITY

Eicosanoids[140] and PUFAs have also been implicated in male reproductive function. AA itself, as well as prostaglandins and leukotrienes, have been implicated in mediating the stimulatory actions of luteinizing hormone on testicular steroid synthesis,[141–143] where AA release is effected through activation of PLA_2.[144]

n-3 PUFA (linolenic acid) supplementation in the diet caused a marked decrease in testicular size and loss of fertility, whereas n-6 PUFA (linoleic acid) supplementation had no effect on testis size or fertility.[145] The decrease in testicular size was found to be due to a degeneration of seminiferous tubules and loss of germ cells associated with an absence of spermatozoa. Interestingly, there was no change in Leydig cell number.[145] Addition of AA (n-6 PUFA) to Leydig cells caused an increase testosterone output[107] which was antagonised by EPA (n-3 PUFA) even though PGE_3 (a product of n-3 PUFA oxygenation) was found to be capable of stimulating steroid synthesis.[107]

In mammals, the lipid composition of sperm membranes plays a major role in the physicochemical modifications leading to fertilization.[146] In all species, phospholipids are the major lipid components of spermatozoa and they contain large amounts of PUFAs.[147–149] Fowl fed on diets containing different compositions of n-3 or n-6 PUFAs yielded sperm containing altered PUFAs in membranes. This suggested that transfer of PUFAs from diet to sperm is effective. High n-3 PUFA levels in the diet led to increases in n-3 PUFA in sperm membranes and this was associated with an increase in fertilizing ability and semen quality.[53,54]

CONCLUSIONS

The preceding survey has established that changes in dietary PUFA composition can affect membrane phospholipid PUFA content and alter prostaglandin synthesis. It has also illustrated the diverse and profound roles played by eicosanoids in general and prostaglandins in particular, in reproduction and fertility. Moreover, this survey has highlighted the paucity of information pertaining to lipid metabolism in endometrial and ovarian tissues of domestic ruminants during cyclicity or gestation. Our relative lack of knowledge means that it is impossible to predict at present whether particular dietary manipulations, which may be desirable from a human health viewpoint, will enhance or reduce fertility. Therefore, it is essential that further research into this general area is carried out before any changes in feed in terms of PUFA composition, are implemented as the fertility of the UK dairy herd is

already extremely poor with average conception rates of only around 50%.

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